Apple blossom colonisation by *Erwinia amylovora* and activity of antagonists at high spatial resolution

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Abstract

Despite decades of study and considerable progress made towards understanding the plant disease fire blight, and its causative agent the bacterial pathogen *Erwinia amylovora*, we still have no effective disease management system. Antibiotics, while initially an effective solution have proved unsustainable due to the increasing prevalence of antibiotic resistant strains of *E. amylovora*. Biological control offers an alternative treatment and prevention option by taking advantage of the natural antagonism between environmental bacterial strains. Identifying the bacterial strains that offer protection to apple blossoms and how they affect the infection process of *E. amylovora*, is the first step in developing a biological control system to protect the billion dollar global apple industry.

The research described in this thesis demonstrates the changing growth of *E. amylovora*, measured by both population size and with microscopic investigations of fluorescently labelled bacterial strains on apple blossoms. This research will identify strains effective in the management of *E. amylovora* infections, and develop a better understanding of the antagonistic mechanisms utilised by biocontrol strains for fire blight disease control.

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Chapter 1 Introduction to fire blight

1.1 Fire Blight Disease Overview

Fire blight is a bacterial disease capable of decimating apple and pear orchards and a universal problem for production of pome fruits including apples. Fire blight disease is caused by the pathogenic bacteria *Erwinia amylovora* that under specific circumstances is able to enter plants through wounds or more commonly the nectarthodes of blossoms [1]. The pathogenic bacteria moves through the trees reaching the twigs, branches and occasionally the trunk resulting in the death of the tree [2]. In cases where only blossom blight occurs, the fruit crop for that year will be lost, while a severe outbreak in which infections spreads to the tree trunk takes years for an orchard to recover from, with lasting consequences for the production of fruit, and the economy that their cultivation supports.

Fire blight is thought to have evolved on indigenous American plants such as Hawthorn and crab apple and may have had an advantageous effect as a 'natural pruning' method with the occasional damaged branch invigorating the plant leading to increased production of fruiting wood [2]. On plants with greater susceptibility fire blight leads to progressive necrosis and death [3].

Fire blight was first observed in the late 18th century and has now been reported in over 40 countries [2]. In 1870 fire blight was recognised as a major problem for production of fruit especially pears and apples [2]. It is believed that human activity including migration and cultivation of susceptible species has been an important factor in the spread of fire blight [2].

Fire blight is believed to have entered New Zealand on imported nursery stocks, with the first outbreak observed in 1919 around Auckland in pear, quince, hawthorn and apple plants [2]. Eight years after fire blight was first reported in New Zealand, the disease caused severe losses to most of the apple cultivars in the Hawkes Bay region [2]. Despite strict regulations on the movement of plant material and bees from infected areas, fire blight reached the South Island in 1929 [2]**.** In 1936, 17 years after first arriving in New Zealand, the disease was detected in the lower South Island in the Otago region, having migrated down nearly the entire country [2].

1.2 Economic costs due to fire blight

The economic cost of fire blight is difficult to determine as small losses are often not recorded while a severe epidemic can disrupt orchard production for several years [4–6]. The continuing spread of fire blight as well as the lack of effective methods for control, leaves us unable to prevent severe outbreaks and economic losses [4]. Fire blight has quarantine status outside of North America, interfering with trade to unaffected countries such as Australia and Japan [4,7]. New Zealand has been named as the words most competitive apple industry from 2015 to 2018 when compared with 33 major apple growing countries [8]. New Zealand's apple industry is worth $850 million dollars (NZD). In 1998 damage due to fire blight in the Hawkes Bay region of New Zealand was estimated to be at least $10 million (NZD) [2]. New Zealand has an ideal climate for apple trees, with plenty of sunshine and temperate seasons due to the surrounding Pacific Ocean, with many popular varietals exhibiting increased sensitivity to fire blight [9–11].

Despite New Zealand's successes, import of New Zealand apples into Australia has been prohibited prohibited since 1919 due to the risk of fire blight transmission [12,13]. After three requests to import in 1989, 1995, and 1999, permission was granted in 2011 provided strict measures are put in place to monitor and restrict fire blight [12,13]. Export of apples from New Zealand to Australia has only occurred due to the intervention of the World Trade Organisations Dispute Settlement Process (WTO, DSP) who declared that Australia's trade restrictions were in breach of the Agreement on the Application of Sanitary and Phytosanitary Measures (SPS Agreement) [12]. Australia has since appealed this decision demonstrating how strongly Australia is opposed to risking fire blight affecting its own apple and pear industry [12].

1.3 Symptoms and disease progression

Fire blight is easily identifiable by its characteristic symptoms of water soaking, wilting and necrosis. Water soaking results from movement of bacteria into the tissues and excretion of bacteria embedded in exopolysaccharides [2]. It has been hypothesized that increased water resulting from rain, dew or humidity promotes excretion of embedded bacteria by hydrating the exopolysaccharides allowing them to expand [2]. Fire blight associated wilting is a consequence of the parenchyma collapsing and loss of plant cell turgor [2]. Progressive necrosis as the disease progresses maps the movement of *E. amylovora* through the host plant [2].

Cankers provide a primary inoculum source of *E. amylovora*, secreting a bacterial ooze with viable bacteria embedded in a hygroscopic polysaccharide matrix [2,14–16]. Ants, flies and rain act as vectors for the bacteria, moving them from canker ooze to blossoms [17–21]. Practically it is extremely difficult to locate and remove every canker so canker removal alone is not sufficient for prevention of fire blight outbreaks [2]. Successful blossom infection from canker

ooze is determined by three major risk factors; insects, temperature, and rain or heavy dew [17–20,22,23]. Blossom infection is the most common site of entry into apple trees and prevention of blossom blight is an important aspect of fire blight control [24–26]. As the disease progresses, the blossom stem exudes bacterial ooze, providing a secondary source of inoculum which can further propagate the disease [27]. Honey bees and other flower- foraging insects are regarded as key vectors for blossom to blossom transmission of bacteria [23,28,29]. The apple blossom as a site for fire blight prevention will be the focus of this research.

There is no agreed theory as to how *E. amylovora* migrates through plant tissue to infect the entire tree but the process may differ depending on the site of initial colonisation [2]. The only consensus regarding *E. amylovora* infection is that the bacteria do not produce enzymes that degrade plant cell walls so it is concluded that internal bacterial migration is dependent on pre existing plant structures [2,30]. There are two competing theories regarding the movement of *E. amylovora* through plant tissue; either *E. amylovora* enters and travels through the plant xylem, or *E. amylovora* enters the cortical parenchyma, possibly from the xylem, and travels through the plant [31,32]. There are many questions remaining about *E., amylovora* migration through plant tissue and they remain interesting topics for future investigation but they are not within the scope of this project.

1.4 The apple blossom as a habitat for bacteria and development of blossom blight

The focus of this research will be the apple blossom as both a favourable habitat for *E. amylovora*, and as a key to fire blight management. Apple blossoms are the primary site of entry for *E. amylovora* into the tree and therefore provide a target for the prevention of the fire blight disease.

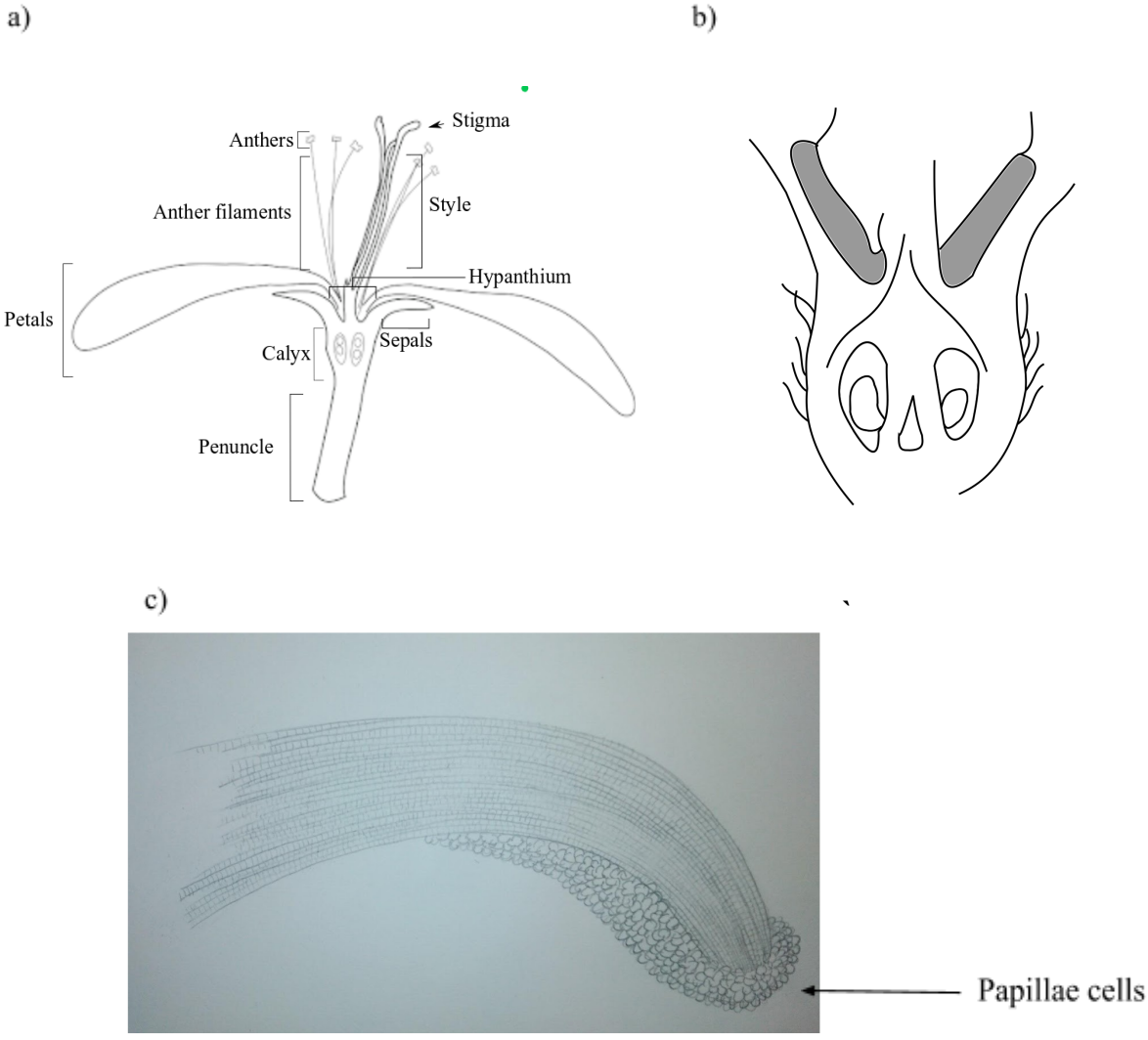

Figure 1. Apple blossom morphology. a) shows the complete blossom labelling all major organs. b) shows a close up of the hypanthium with the nectarthodes shown in grey, located between the style and sepals. c) shows a blossom stigma. The side with papillae cells is the inside of the stigma/ style, while the outer side does not have papillae cells.

Apple blossoms provide a protective and nutrient rich habitat for a diverse community of microorganisms including bacteria. The bacterial community on apple blossoms is diverse and changes from bud to senescence [33]. Blooming exposes the reproductive organisms to

pollinators, both insect and environmental, pollinators which also facilitate the movement of bacteria between blossoms [33,34]. The stigma supports large communities of microbes relative to the rest of the blossom due to the secretion of sugars and amino acids and is therefore the primary site of *E. amylovora* colonisation (Figure 1) [1,35–38]. While anthers also support an epiphytic population, they are considered less significant because they host smaller populations that are less able to move from the stigma to the hypanthium [25]. Large epiphytic populations of *E. amylovora* 10^5 to 10^7 can be found on blossoms with no symptoms of blossom blight suggesting that *E. amylovora* infections of the apple blossom stigma are not always pathogenic, and in many instances do not interfere with the development of normal fruit [1,39–41]. Previous studies have shown populations of *E. amylovora* on apple blossom stigma favour growth in intercellular spaces of the stigma due to secreted nutrients and high humidity [25,42]. The morphology of stigmas and styles varies between members of the Rosaceae, but they are characterised as having wet stigmas, an environment conducive for *E. amylovora* infection likely contributing to the susceptibility of the Rosaceae [37,43,44]. Rain or heavy dew facilitate the migration of *E. amylovora* from the stigma into the hypanthium [1,25,45]. Rain not only facilitates movement of *E. amylovora* into the hypanthium, but also causes otherwise unsuitable hypanthium surfaces to be viable for bacterial growth [45]. The nectarthode cells provide an opening for the bacteria into the tree, after which blossom blight develops [46]. The rate of symptom development has been found to directly correlate with the number of *E. amylovora* cells introduced to the hypanthium [25,47]. Preventative measures to reduce the stigma population result in fewer bacterial cells descending into the hypanthium, thereby reducing the incidence of blossom blight. Apple blossoms older than three days are unable to support growth of *E. amylovora* which is attributed to collapse of the stigma papillae cells (Figure 1) [48]. The growth of *E. amylovora* in the intercellular spaces of papillae

cells may be an important step in the development of fire blight and the growth to large population sizes required for blossom blight infection [25,47]. The inability of *E. amylovora* to grow on blossoms older than three days in addition to blossom blight development being so heavily influenced by the number of *E. amylovora* cells, provides an obvious target for preventative efforts; stopping *E. amylovora* colonising apple blossoms during the three days following blooming, and minimise their population size.

1.5 *Erwinia amylovora*: understanding the pathogen

Efforts to prevent infection of *E. amylovora* in pear and apple cultivars has yielded no satisfactory management strategy despite the continued research into the bacteria and their activity. *E. amylovora* appears to have the ability to use a diverse range of methods to spread, which may be why fire blight is such a difficult disease to understand and control. *E. amylovora* was the first bacterial plant pathogen to be described in the late 1790's, and is the only bacterium responsible for fire blight disease [4]. The bacteria are mobile by means of peritrichous flagella, and although this is not a requisite for pathogenicity, it enhances the pathogen infectivity [49]. The pathogen has been found to be chemotactically attracted to nectar extracts of apple blossoms [50]. *E. amylovora* populations on the stigma have been shown progress towards the hypanthium via the stylar groove, suggesting a conserved infection pathway [51].

The *hrp- dsp* genes encode functions responsible for secretion of protein factors involved in plant symptom development including electrolyte leakage from plant cells [52–58]. The gene sequences of the European *E. amylovora* CFBP1430S compared to the US *E. amylovora* Ea273 strains have been found to be nearly identical with 99.99% similarity [59]. The low diversity indicates minimal evolution since the global dispersal of the pathogen, therefore

new treatment options can be expected to show similar effectiveness against both strains [59]. Three type VI secretion systems (T6SS) were also identified in the genome of *E. amylovora* CFBP1430S, acting as a targeted antimicrobial harpoon that punctures a target cell to destroy it [59,60].

1.5.1 Iron limitation as a virulence trigger

Iron is a catalyst of many crucial metabolic processes and is therefore essential for all life. Iron is not always readily available for uptake by microbial species, hence why many bacterial species including *E. amylovora* produce siderophores, molecules with a high affinity for iron in both organic and mineral substrates as a means to overcome iron limitation [61,62]. Under conditions with low iron availability, the African violet pathogen *Erwinia chrysanthemi* 3937 undergoes the coordinated activation of multiple virulence pathways [63,64]. Apple blossom stigma have limited iron availability to microbes therefore understanding the potential role of iron limitation in both the activation of *E. amylovora* virulence, and as a disease control is a strategy with considerable potential [61,62,65]. The growth of *E. amylovora* extracellularly on tissue with slow cell death in addition to the inability to degrade cell walls provides a situation in which iron competition is important for success [2,62].

Under limited iron conditions the ferric uptake regulation (Fur) regulates synthesis and uptake of siderophores. *E. amylovora* is known to only produce the hydroxamate siderophore desferrioxamine (DFO) E [62,66]. Transposon mutants with a compromised *dfoA* gene are strongly affected in their ability to colonise apple blossoms and initiate necrosis, showing decreased potential for virulence [67]. Iron competition is expected to play an important role in bacterial interactions on apple blossoms and will be discussed further in section 1.6.

1.5.2 Antibiotic resistance of *E. amylovora*

Chemical control of bacterial pathogen can be achieved by targeting and eliminating bacteria, or by rendering the environment in which they grow unsuitable [68]. Chemical control of fire blight is used to destroy overwintering sites, or to protect invasion sites such as the apple blossom [68]. Copper compounds have previously been used as a control method but their phytotoxic effect on the plant has led to their discontinued use in favour of antibiotic compounds [68]. *E. amylovora* was among the first bacterial diseases to be tested against antibiotics, although only streptomycin and to a limited extent oxytetracycline and kasugamycin were found to be both effective in controlling field populations as well as being non toxic to plants [68]. Streptomycin is produced by *Streptomyces* spp. and targets a broad range of Gram negative and Gram positive bacteria [69]. Streptomycin binds within the small ribosomal subunit inhibiting protein synthesis [70,71]. By the late 1960s streptomycin had been deployed for the management of fire blight [72]. *E. amylovora* was the first bacterial strain detected with streptomycin resistance, resistance which does not reduce the risk of blossom blight but does render most treatments ineffective due to the continued reliance of fire blight control strategies entirely or partially on streptomycin [29,68,73,74].

Streptomycin resistance can be achieved through a variety of mechanisms, but the majority of streptomycin resistant strains bear resistance due to genes encoding enzymes that inactivate the streptomycin molecule [75]. Globally the two most widely distributed resistance factors are the *strAB* gene pair (*strA-strB*) and the *aadA* gene. The *strAB* pair is associated with the transposon Tn5393 and with small broad host range plasmids [76]. The *aadA* gene is associated with integrons, mobile genetic elements with the ability to acquire additional resistance genes [77,78]. As resistances commonly occur on mobile genetic elements, resistance can easily be

spread between bacterial cells [69]. Spontaneous mutations in the *rrs* or *rpsL* genes also impart resistance by altering the ribosomal target site [79]. Point mutation is associated with resistance to higher concentrations compared to acquired resistance making growth in concentrations of streptomycin up to 4,096 ppm possible [72,74,80,81]. The resistance observed in bacterial pathogens is the result of: escape of naturally occurring resistance genes through horizontal gene transfer, the removal of resistance genes present within the microbiome to other strains under conditions of antibiotic selection, and mutations resulting in alterations to antibiotic target sites [69]. Even in orchards in which streptomycin use has been discontinued, streptomycin resistant strains of *E. amylovora* are still detected many years later [82,83]. Global prevalence of streptomycin resistant *E. amylovora* strains in addition to restrictions on the use of antibiotics makes finding sustainable and effective solutions to prevent fire blight a pressing issue [72,83,84].

1.6 Biocontrol of *E. amylovora*: an imperfect alternative

The use of antagonistic strains for the control of fire blight is an emerging sustainable albeit imperfect method for fire blight control [85–88]. Biological control of fire blight exploits existing competition or production of inhibitory compounds by an antagonistic bacterial strain to prevent establishment and growth of *E. amylovora* on apple blossom stigma [25,89]. Reducing the population of *E. amylovora* on apple blossoms stigmas has a two fold effect in reducing fire blight occurrence though a) reducing the population size on apple blossoms and therefore the risk of blossom blight and b) reducing the likelihood of transfer to an uninfected blossom through rain or insects [90–93]. Bacterial strains that occupy the same stigmatic intercellular spaces as *E. amylovora* and have similar requirements for essential nutrients are expected to impose limitations on pathogen growth [37,94]. In addition to nutrient exclusion, secretion of

compounds such as siderophores or antimicrobial molecules can affect survival of *E. amylovora* [65,88,95,96]. For the remainder of this thesis, antagonism will be used to refer to any inhibitory effect by an environmental strain regardless of the mechanism by which this is achieved.

1.6.2 *Pantoea vagans* C9-1: a known antagonist

Pantoea vagans C9-1 (*P. vagans*) is a known antagonist of *E. amylovora* registered as a fire blight control product under the name BlightBan C9-1 (Nufarms America Inc., Burr Ridge, IL). Although slightly less effective than streptomycin, *P. vagans* has been shown to be an extremely effective antagonist of *E. amylovora* [29,85,97,98]. *P. vagans* produces multiple antibiotics, two of which have been identified as pantocin A and dapdiamide E as well as siderophores for increased efficiency of iron uptake [67,97,99–102]. Pantocin A is a small peptide antibiotic requiring a nitrogen poor environment to be uptaken into the target cell via tripeptide transporters, where blocks an enzyme required for histidine synthesis therefore pantocin A effects can be alleviated by supplementary L- histidine [99,100,103,104]. As the stigma is known to secrete amino acids, the blossom environment may render pantocin A less effective [35].

Although it is possible for *E. amylovora* to develop spontaneous resistance to antibiotics produced by *P. vagans*, the biocontrol strain is predicted to still have an inhibitory effect against *E. amylovora* [101]. *P. vagans* produces multiple siderophores, with desferrioxamine E being the major product [105,106]. The genome of *P. vagans* C9-1 encodes ten TonB- dependent receptors, while the genome of *Erwinia* spp. only encodes four which Smits et al. (2011) hypothesise results in *P. vagans* having a more efficient iron uptake strategy than *E. amylovora* [59,88,107,108]. Within the genome of *P. vagans*, two type VI secretion system gene

clustered have been identified [59,88]. As with most biocontrol strains preemptive exclusion of colonisation sites is important for *P. vagans* success in inhibiting *E. amylovora* [96]. The sugars secreted by apple blossoms and utilised by *P. vagans* are believed to overlap with the requirements of *E. amylovora* [85,109,110]. Apple blossom stigma infection by *P. vagans* was shown to follow the same physiological process as *E. amylovora*, moving down towards the hypanthium via the stylar groove, evidence that spatially and nutritionally *P. vagans* has many similarities with *E. amylovora* in apple blossom stigma colonization [51]. *P. vagans* has multiple mechanisms by which antagonism of *E. amylovora* can be mediated, making it a highly successful biocontrol strain.

1.6.3 *Pantoea agglomerans* 299R

Pantoea agglomerans 299R (*P. agglomerans*) has been intensively studied as a model phyllosphere bacteria [96,111–114]. *P. agglomerans* 299R does not produce any antibiotics, although many other *Pantoea agglomerans* spp. do produce at least one if not multiple antibiotics, with encoding genes localized on the chromosome as well as plasmids depending on the strain [11597,103,115–122]. Strains of antibiotic producing *P. agglomerans* strains have been shown to reduce development of fire blight even when antibiotic production is disrupted, but this result is not consistently observed, and the level of inhibition is not as strong as observed for antibiotic producing strains [118118,123].

Similarly to *P. vagans*, *Pantoea agglomerans* strains Eh252 and Eh159 (Eh252 and Eh159 respectively) were shown to have similar colonization events as *E. amylovora* 273 [37]. Given the low variability in strains of *E. amylovora* after global dispersal, the stigma colonization events of *E. amylovora* 273 are expected to be similar to *E. amylovora* CFBP1430S, favouring areas of the stigmatic surface with deteriorating cuticle, the layer encasing the

papillae cells [37,59]. Different strains of *P. agglomerans* were found to vary in their colonization process, with Eh252 showing greater similarity to *E. amylovora* 273 compared to Eh159, as well as greater biocontrol ability [37]. *P. agglomerans* is also known to have genes encoding a T6SS which may function as a mechanism of antagonism against *E. amylovora* [124]. In this research I will be investigating whether *P. agglomerans* 299R strain shows similarity in colonisation process or nutritional requirements to *E. amylovora*. This will evaluate whether this strain has potential as a biocontrol agent of *E. amylovora*.

1.6.4 *Pseudomonas fluorescens* PFO-1

Pseudomonads are commonly associated with the Rhizosphere supporting plants through nutrient cycling and protection against pathogens [125–128]. *Pseudomonas fluorescens* A506 is an established antagonist of *E. amylovora* currently available commercially under the name BlightBan A506 (NuFarm Americas, Burr Ridge, IL [37,129].The *Pseudomonas fluorescens* PF01 (*P. fluorescens*) strain in comparison to A506 has been studied less extensively as a potential antagonist but will be evaluated in this research as a potential antagonist of *E. amylovora*. Members of the *P. fluorescens* spp. are known to produce pyoverdine, a fluorescent pigment which binds and carries free Fe^{3+} acting as a siderophore, under conditions with reduced iron availability [128,130,131]. The role of pyoverdine in limiting iron availability has been linked to control of pathogens in the rhizosphere [128,130]. Iron availability is limited on stigmatic surfaces of apple blossoms, which stimulates the secretion of siderophores by *P. fluorescens* A506 [65]. Production of pyoverdine was found to be inversely correlated with the production of antibiotics, suggesting that antibiotic production on apple blossom stigma by *P. fluorescens* A506 may be limited by iron availability and therefore irrelevant to the biocontrol exhibited by this strain [65]. *Pseudomonas* spp. that produce

siderophores were shown to inhibit the growth of bacteria and fungi less able to bind iron [132,133]. Although comparing the iron affinity of pyoverdine produced by *P. fluorescens* PF01 with desferrioxamine E produced by *E. amylovora* was not undertaken in this study, it is expected that iron availability will be a limiting factor for both of these strains on apple blossom stigma.

1.6.5 *Sphingomonas melonis* Fr1

Sphingomonas melonis (*S. melonis*) is a species often found in the phyllosphere of a variety of plants which has been shown to have a protective effect against the bacterial pathogen *Pseudomonas syringae* on *Arabidopsis thaliana* leaves [65,94,134,135]. *P. syringae* infection has an an epiphytic phase of growth prior to tissue penetration, and a correlation of cell number with likelihood of symptom development [90,94]. *S. melonis* was found to prevent disease symptoms and kept the cell numbers of *P. syringae* at low numbers [94]. Innerebner et al. (2011) concluded that considerable overlap in nutritional requirements and high cell numbers of *S. melonis* which exclude the pathogen from leaf sites are the means by which *S. melonis* inhibits *P. syringae* [94]. No antagonistic potential of *S. melonis* against *E. amylovora* on apple blossoms have been found, however given the broad range of hosts on which *S. melonis* thrives, it seems likely that the apple blossoms would provide a suitable habitat for this strain, thereby excluding *E. amylovora* as a method of biocontrol.

1.7 Thesis outline and hypotheses

The focus of previous research into *E. amylovora* and its antagonists has primarily focussed on populations as a whole. Although minimising *E. amylovora* population sizes is important for successful biocontrol, less emphasis has been given in considering the apple blossom

itself and how bacteria utilise this niche. Much can be learned by studying bacterial populations as a whole, in a heterogeneous environment such as an apple blossom, the average does not represent how each individual cell is thriving [136]. By using high resolution fluorescence microscopy, this research will generate a detailed and comprehensive understanding of how *E. amylovora* populations colonise the apple blossom stigma and how antagonistic bacterial strains disrupt this process. Colonisation of *P. vagans, P. agglomerans*, *P. fluorescens* and *S. melonis* will be compared with *E. amylovora*, and strains with similar population development processes to *E. amylovora* are expected to be successful antagonists [37,94,96].

My research hypotheses are as follows:

1) The four strains selected for this study will show an effect in inhibiting the growth of *E. amylovora*.

2) On any given surface, both artificial and plant, preemptive colonization will determine antagonistic success.

3) *E. amylovora* will impair the growth of antagonistic and potentially antagonistic strains when applied to surfaces, both artificial and plant.

4) Strains with an antagonistic effect against *E. amylovora* will colonise apple blossoms in the same way as the pathogen.

For this research growth refers to all indications of population success; increase in colonised area on solid media, increase in cell number, and colonisation of new territories on apple blossoms. Chapter 2 investigates the comparative growth of *E. amylovora* when applied onto solid media. The control growth will be compared to *E. amylovora* growth when a secondary

strain is applied either simultaneously or delayed onto the media after *E. amylovora* inoculation. These trials will test the first two hypotheses. Conversely the antagonistic and potentially antagonistic strains will be investigated to see if their growth on solid media is affected by delayed or simultaneous application of *E. amylovora* in order to test my third hypothesis.

Chapter 3 evaluated changes in the population size of *E. amylovora* when inoculated with an antagonist or potential antagonist. The antagonist cell numbers were also investigated for any changes when inoculated with *E. amylovora* rather than individually. Chapter 3 further investigates hypotheses 1 and 3, and although the findings may support conclusions regarding pre colonisation by antagonists as an important determinant of biocontrol, investigation of delayed application was not undertaken, so only partially addresses hypothesis 2 [137,138]. Chapter 4 addresses hypotheses 1 and 4. The spatial distribution of fluorescently tagged strains was investigated using microscopy techniques to further understand how colonisation of the apple blossom stigma by antagonistic strains occurs compared to colonisation by *E. amylovora*, and to investigate whether stigma infection by *E. amylovora* is disrupted by antagonistic strains. Chapters 3 and 4 will generate a comprehensive understanding of population growth with regards to population size and colonisation events on the apple blossom stigma and style.

Chapter 2 *In vitro* investigations of antagonists effect on growth of *E. amylovora*

2.1 Introduction

2.1.1 In vitro studies to inform initial understanding of bacterial interactions

An effective and sustainable method of fire blight control is no closer to completion than it was when fire blight was first recognised as a disease in pomme fruits. Fire blight and the causative bacterial agent *E. amylovora* continue to be studied around the world in an effort to develop methods for the prevention and control of fire blight that do not rely on antibiotics [29,86,123,138,139]. This chapter will focus on our *in vitro* studies towards further understanding how antagonists affect growth of new and established *E. amylovora*. Better understanding of antagonistic bacterial strains and their influence on the growth of *E. amylovora* is crucial in optimising their use for prevention and management of fire blight.

Inoculation studies that apply bacteria to apple blossoms, whether on the tree or detached, facilitating a blossom blight infection best represent disease manifestation naturally in orchards and in the wild [2]. However blossom inoculation has no surety that inoculated blossoms will yield blight in woody tissue limiting study options to non-woody tissue. Additionally, the blossom production season is short limiting the time to conduct studies using apple blossoms. It has been established hat 4-5 days after blossoms have opened, *E. amylovora* is unable to grow or has very limited growth on stigmas of royal gala apple blossoms [140]. Artificial inoculation can be achieved by wounding the trees, which allows for disease to be initiated year round without the need for blossoms and is ideal for studying disease in woody tissue, although this method may allow *E. amylovora* to enter tissue it

would not usually during blossom infection so is not truly representative of natural infection through the blossom nectarthodes [2]. Studying bacteria *in vitro*, while not representative of infection of apple blossoms, does eliminate the difficulties incurred by working with apple blossoms and apple trees.

As this study is interested in the initial events of *E. amylovora* colonisation we only monitor the first four days of bacteria growth. Any studies beyond four days of interactions would investigate activities that apple blossoms with their limited viability are not capable of hosting, so are therefore of lesser interest and not within the scope of this study. I have set out to study if the bacterial strains *P. vagans*, *P. agglomerans*, *P. fluorescens*, or *S. melonis* are able to treat as well as prevent growth of *E amylovora*. If *E. amylovora* is applied embedded in a solid media layer, can applied strains counteract existing growth and prevent new growth? In contrast can *E. amylovora* still successfully colonise an area despite an antagonistic strain already being established in a solid media layer indicating susceptibility of the antagonist to *E. amylovora*?

Survival of two bacterial strains in the same environments can be achieved by: strains colonising a habitat at different times, spatial segregation, or strains having different nutritional requirements with little overlap. In my experiments, I am forcing the strains to be present at the same time and as we are applying the strains on top of each other, a spatial segregation is not possible unless one strain eliminates the other from an area. The double layer assay assesses the similarity of *E. amylovora* nutritional requirements compared to the competing strains. *P. vagans*, *P. agglomerans*, and *P. fluorescens* secrete antibiotics, siderophores, or cell targeting attack machines so I infer that these strains will show greater antagonism than *S. melonis* [88,108,141–144].

2.1.2 The double layer assay

The double layer assay has previously been used to detect antagonistic interactions of *Pseudomonas orientalis* F9 against *E. amylovora* [145]. The double layer assay embeds a strain into a soft tryptic soy agar layer, followed by application of a second strain on top as droplets of bacterial suspension. Prior studies have used similar techniques to investigate the coexistence of epiphytic bacterial populations and the role nutritional resource partitioning has in species interaction [96,146]. Visualising antagonism against *E. amylovora in vitro* by *P. vagans, P. agglomerans, P. fluorescens*, and *S. melonis* sets a precedent for expected interactions on apple blossoms and informs the development of the experiments described in the following chapters.

2.2 Materials and Methods

The following methods are based on the methods used by Zengerer *et al*. 2018.

2.2.1 Double Layer Assay

E. amylovora and a competitor strain (*P. vagans* C9-1*, P. agglomerans* 299R*, P. fluorescens* PF0-1 or *S. melonis* Fr-1) were cultivated on tryptic soy agar (TSA, HiMedia) plates at 30° C overnight (approximately 15 hours). Freshly grown bacterial colonies were suspended in 1 × phosphate buffered saline (1 × PBS, 8 g/ L NaCl, 0.2 g/ L KCl, 1.4 g/ L Na_2HPO_4, 0.2 g/ L KH_2PO_4, prepared by diluting 10 × PBS), and adjusted to OD_{600nm} of 1.0. 300 uL of the resuspended culture was diluted with 30 mL of molten soft TSA (15 g/ L tryptone, 5 g/ L bacteriological peptone, 5 g/ L NaCl, 7.5 g/ L agar bacteriological) cooled to 40° C celsius in a 50 mL Falcon tube. Initial experiments demonstrated that in order to cool agar to a low enough temperature for addition of bacteria, making the agar in alioquotes of 250 mL of less was best. Larger volumes of agar maintained too much heat even when cooled and significantly decreased the viability of added bacteria. The Falcon tube was gently inverted three to four times to resuspend the bacterial suspension and 10 ml of the soft TSA-bacterial suspension were poured onto a TSA plate. The soft TSA was allowed to set in a laminar flow for 20 minutes. A total of nine plates with *E. amylovora* diluted with soft TSA, and nine plates with a competitor strain diluted with soft TSA were prepared. Of the nine plates prepared with an embedded strain, three were kept as controls and received no further treatment, three had a competitor strain applied immediately after the soft TSA had set, and three had a competitor strain applied after 48 hours of incubation as described below.

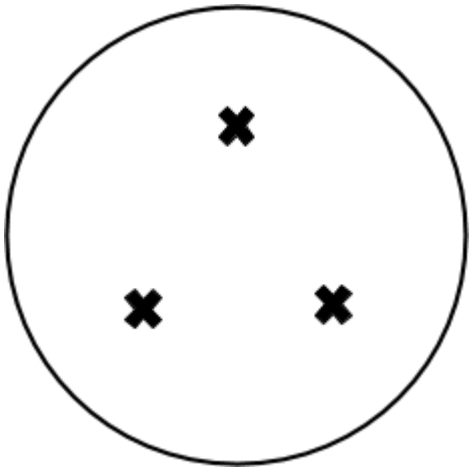

Figure 1 The template used to apply 20 uL inoculation droplets. The 'X' indicates where a 20 uL droplet was applied to the plate.

Competitor strains were prepared using fresh colonies suspended in 1 × PBS and adjusted to an OD_{600nm} = 1.0. 20 uL droplets of the suspension were applied using the template shown in Figure 1 and allowed to dry for 20 minutes under a laminar flow. All plates were allowed to incubate at 30 degrees. The plates were photographed as explained below.

2.1.2 Photography

For each growth spot, a scale of 10 mm printed on paper was applied to the surface of the agar next to the colony to measure the diameter of each growth colony. Each of the nine replicate colonies was numbered. Several different lighting techniques were used to photograph the growth of applied 20 uL spots: Technique 1 involved raising the plate ~3 cm above a white light source. To best display the colonies particularly the ones that had very little difference from the surrounding agar growth, the raised plate had a square of black paper was placed underneath the plate below the colony, which resulted in a darkfield illumination style of photography [147]. Each colony was photographed individually. Technique

2 used two gooseneck lamps positioned to illuminate the colony from opposite sides. This best showed subtle differences in the appearance of the applied strain compared to the appearance of the strain embedded in the media. This technique revealed colonies that were not visible under normal light or using technique 1. The growth of the 20 uL applied strain, and corresponding locations on the control plates were scraped off with a chemical spatula (approximately 2 mm thick, and 15 mm wide) to view the agar underneath and a square of black paper was placed so that it was partially under the space occupied by the 20 uL droplet. The clarity of the paper edge in the droplet area best showed how opaque the agar was underneath. The growth spots were then photographed using technique 1. From the nine replicate droplets applied, a representative image of each was selected and processed using Fiji [148].

2.3 Results

The double layer assay demonstrates competitive interactions between different bacterial strains on TSA media. A positive result was indicated by the presence of a halo. Halos are identified as a clearing of the agar underneath where the 20 uL droplet of a strain was applied onto inoculated soft TSA. Halos were visible after a chemical spatula is used to remove the overlying droplet growth.

2.3.1 *E. amylovora* and *P. vagans*

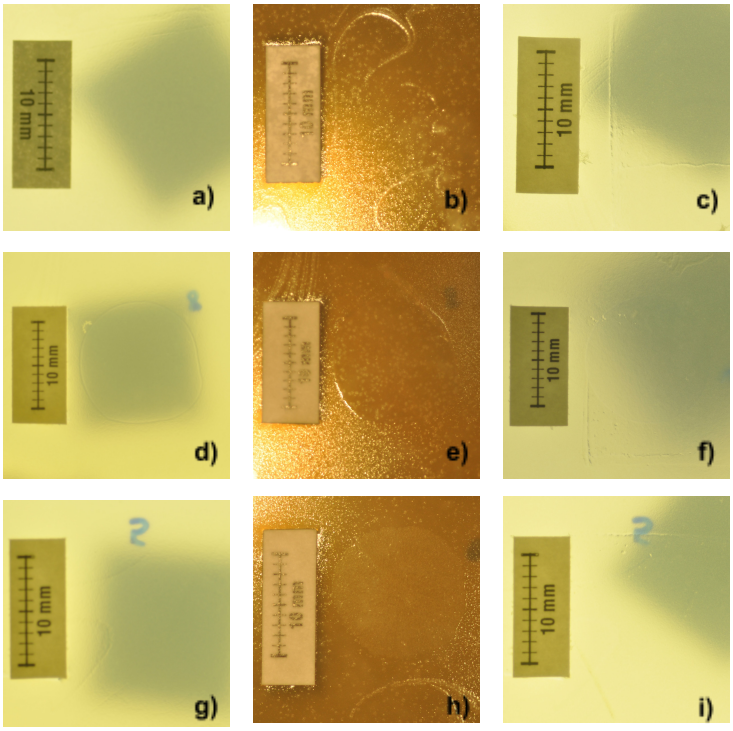

Figure 2. Double layer assay with *P. vagans* in the underlying agar with *E. amylovora* applied as 20 uL droplets. a), b), and c) are the results of *P. vagans* controls d), e), and f) are the results of *P. vagans* with *E. amylovora* applied on top. g), h), and i) are *P. vagans* with *E. amylovora* applied on top after a 48 hour delay. a), d), and g) were photographed using technique 1, b), e), and h) were photographed using technique 2, and c), f), and i) were photographed using technique 1 after colonies had been removed.

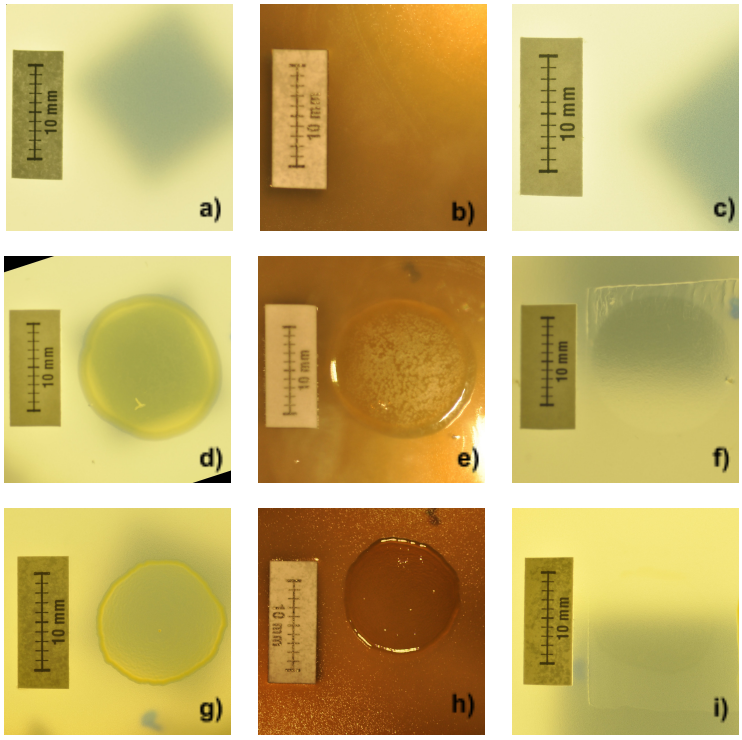

Figure 3. Double layer assay with *E. amylovora* in the underlying agar with P. vagans applied as 20 uL droplets. a), b), and c) are the results of E. amylovora controls d), e), and f) are the results of E. amylovora embedded in the media with P. vagans applied on top. g), h), and i) are the results of E. amylovora embedded in the media with P. vagans applied on top after a 48 hour delay. a), d), and g) were photographed using technique 1, b), e), and h) were photographed using technique 2, and c), f), and i) were photographed using technique 1 after colonies had been removed.

The double layer assay results investigating how *E. amylovora* is able to grow on solid media embedded with *P. vagans* show no halos and therefore no positive result (Figure 2). The lighting technique using gooseneck lamps shows patterns not visible under the other technique. The circle seen in picture e) we believe is an *E. amylovora* colony due to it's raised appearance. In comparison, when *E. amylovora* is applied with a delay, we do not see a

raised colony, just a different texture to the surface so we believe picture h) shows a remnant of the applied spot, but not a colony. When *P. vagans* is applied to media with embedded *E. amylovora* we see strong growth of *P. vagans* under both lighting techniques (Figure 3). Picture f) within this figure shows a halo, the positive result supports the hypothesis that *P. vagans* is able to prevent the growth of *E. amylovora*. When the *P. vagans* is delayed in it's application onto *E. amylovora* embedded media, we see growth under both lighting techniques and a clearing of the agar (pictures g), h), and i), Figure 2). The agar clearing is not complete, but we do see evidence that *P. vagans* is able to affect the growth of pre existing *E. amylovora* colonies.

2.3.2 *E. amylovora* and *P. agglomerans*

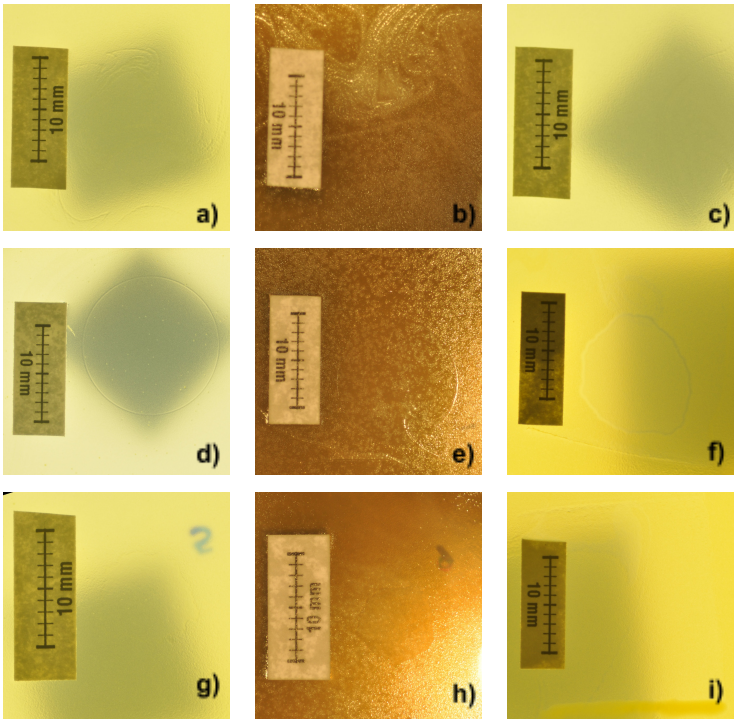

Figure 4. Double layer assay with *P. agglomerans* in the underlying agar with *E. amylovora* applied as 20 uL droplets. a), b), and c) are the results of *P. agglomerans* controls d), e), and f) are the results of *P. agglomerans* with *E. amylovora* applied on top. g), h), and i) are *P. agglomerans* with *E. amylovora* applied on top after a 48 hour delay. a), d), and g) were photographed using technique 1, b), e), and h) were photographed using technique 2, and c), f), and i) were photographed using technique 1 after colonies had been removed.

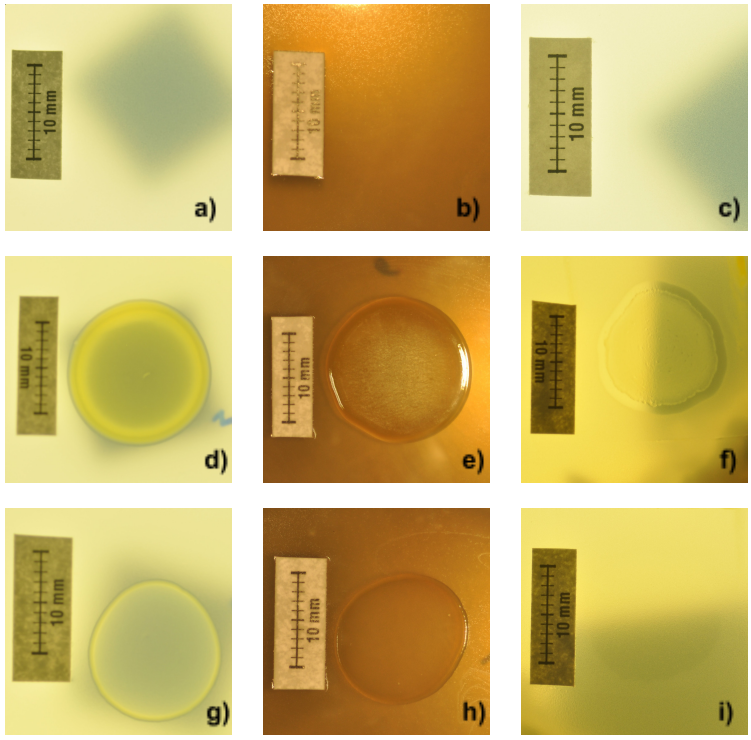

Figure 5. Double layer assay with *E. amylovora* in the underlying agar with *P. agglomerans* applied as 20 uL droplets. a), b), and c) are the results of *E. amylovora* controls d), e), and f) are the results of *E. amylovora* embedded in the media with *P. agglomerans* applied on top. g), h), and i) are the results of *E. amylovora* embedded in the media with *P. agglomerans* applied on top after a 48 hour delay. a), d), and g) were photographed using technique 1, b), e), and h) were photographed using technique 2, and c), f), and i) were photographed using technique 1 after colonies had been removed.

When *E. amylovora* is applied without delay over media with embedded *P. agglomerans* we see some growth of *E. amylovora* as seen by the raised appearance of the colony (picture e), Figure 4). When this growth is scraped off, we see a small outline of cleared agar in a coffee ring effect. There is no observable growth when application of *E. amylovora* over *P.*

agglomerans is delayed by 48 hours. We see no halos formed under *E. amylovora* regardless of whether application of *P. agglomerans* embedded media is immediate or delayed. *P. agglomerans* applied over *E. amylovora* embedded in solid media shows strong growth, evident by the thick raised colonies (pictures d), e), g), h), Figure 5) When the colony resulting from *P. agglomerans* applied with no delay is removed, we see a coffee ring effect of cleared agar with remaining growth in the centre of the ring as seen in picture f), Figure 5 [149,150]. When application of *P. agglomerans* is delayed by 48 hours, we see incomplete agar clearing. Some growth remains under the *P. agglomerans* colony after it has been removed, but this growth is reduced in density compared to the surrounding media.

2.3.3 *E. amylovora* and *P. fluorescens*

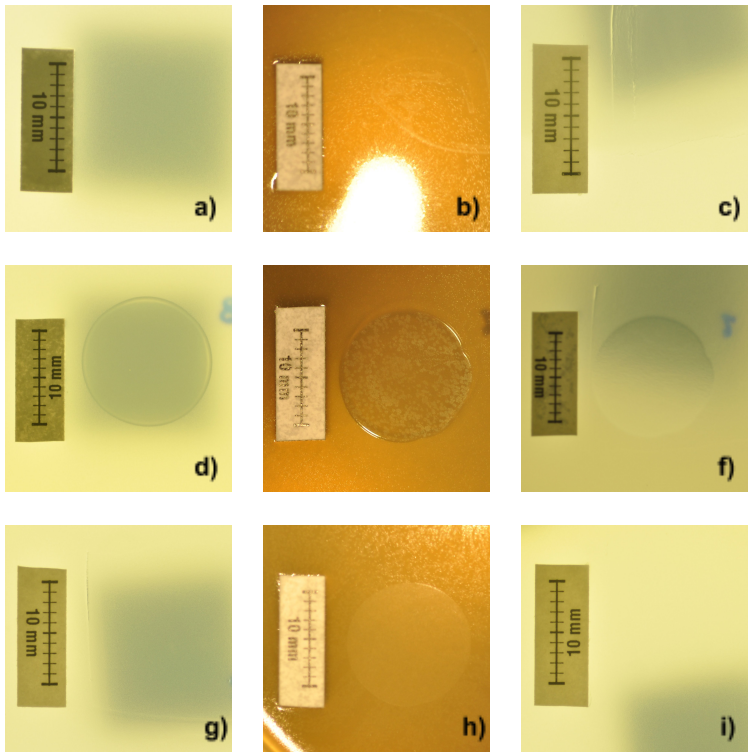

Figure 6. Double layer assay with *P. fluorescens* in the underlying agar with *E. amylovora* applied as 20 uL droplets. a), b), and c) are the results of *P. fluorescens* controls d), e), and f) are the results of *P. fluorescens* with *E. amylovora* applied on top. g), h), and i) are *P. fluorescens* with *E. amylovora* applied on top after a 48 hour delay. a), d), and g) were photographed using technique 1, b), e), and h) were photographed using technique 2, and c), f), and i) were photographed using technique 1 after colonies had been removed.

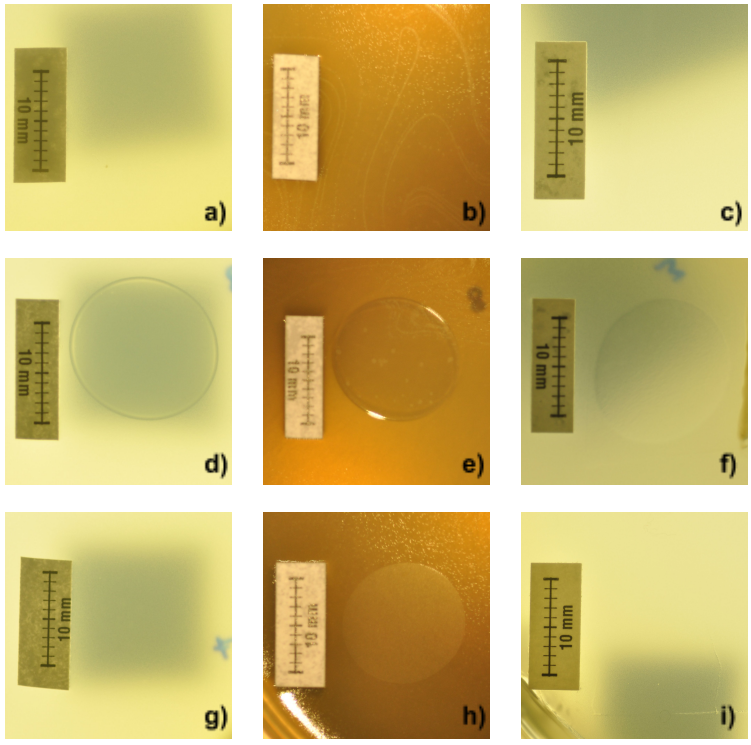

Figure 7. Double layer assay with *E. amylovora* in the underlying agar with *P. fluorescens* applied as 20 uL droplets. a), b), and c) are the results of *E. amylovora* controls d), e), and f) are the results of *E. amylovora* embedded in the media with *P. fluorescens* applied on top. g), h), and i) are the results of *E. amylovora* embedded in the media with *P. fluorescens* applied on top after a 48 hour delay. a), d), and g) were photographed using technique 1, b), e), and h) were photographed using technique 2, and c), f), and i) were photographed using technique 1 after colonies had been removed.

We see a visibly raised colony of *E. amylovora* growing on solid media with embedded *P. fluorescens* which when removed shows a halo of clear agar (e), f), Figure 6). When *E. amylovora* application is delayed by 48 hours, we see some growth as indicated by the raised

appearance, but we see no agar clearing (h), i), Figure 6). Based on these results we have evidence that *E. amylovora* is able to prevent the growth of *P. fluorescens*, but has no effect on existing populations on TSA media. *P. fluorescens* when applied over *E. amylovora* with no delay shows a raised colony of growth and a clearing of agar underneath giving a positive result (e) f), Figure 7). When *P. fluorescens* application is delayed, we see some growth but no agar clearing (h), i), Figure 7). Based on these results we have evidence that *P. fluorescens* is able to prevent the growth of *E. amylovora* but does not have an effect on existing populations. We noticed that the results show the effect *E. amylovora* has against *P. fluorescens* is the same as the effect *P fluorescens* has on *E. amylovora*. We conclude that these strains are equally as susceptible to each other, and strain dominance is always conferred to the primary coloniser.

2.3.4 *E. amylovora* and *S. melonis*

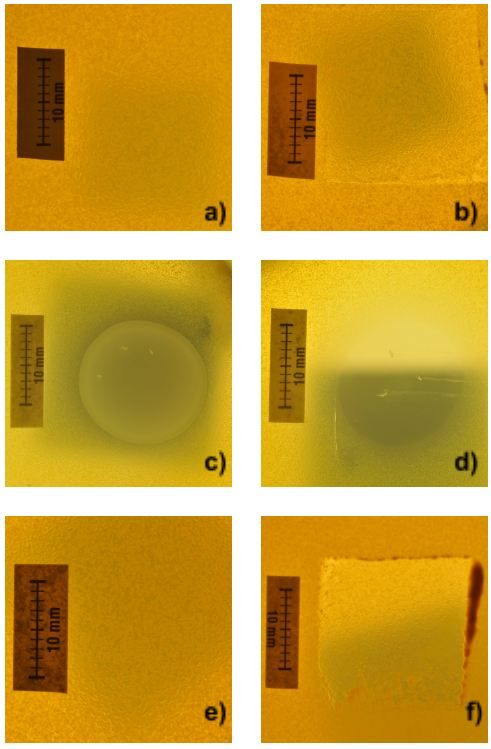

Figure 8. Double layer assay with *S. melonis* in the underlying agar with *E. amylovora* applied as 20 uL droplets. a) and b) are the results of *S. melonis* controls, c) and d) are the results of *S. melonis* with *E. amylovora* applied on top. e) and f) are the results of *S. melonis* with *E. amylovora* applied on top after a 48 hour delay. a), c), and e) were photographed using technique 1, b), d), and f) were photographed using technique 1 after colonies had been removed.

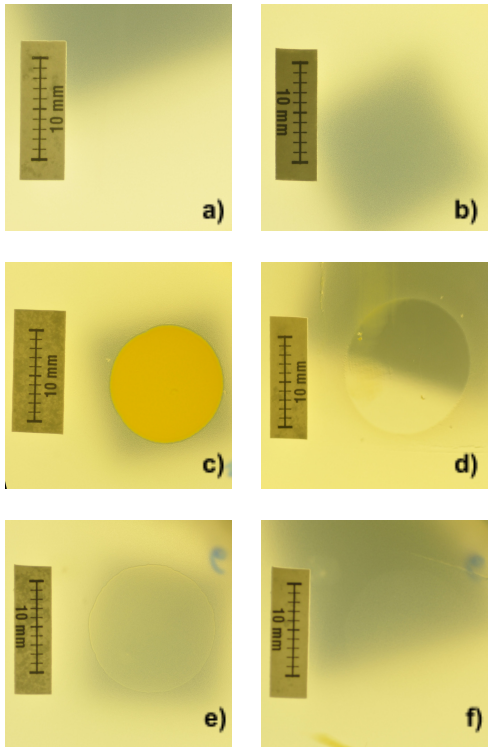

Figure 9. Double layer assay with *E. amylovora* in the underlying agar with *S. melonis* applied as 20 uL droplets. a), b), and c) are the results of *E. amylovora* controls d), e), and f) are the results of *E. amylovora* embedded in the media with *S. melonis* applied on top. g), h), and i) are the results of *E. amylovora* embedded in the media with *S. melonis* applied on top after a 48 hour delay. a), d), and g) were photographed using technique 1, b), e), and h) were photographed using technique 2, and c), f), and i) were photographed using technique 1 after colonies had been removed.

Colonies in our investigations into interactions investigating *E. amylovora* and *S. melonis* were not photographed using two lighting techniques. The significant difference in colour and surface texture between these two strains made identification of each strain easy using the white light background technique. When *E. amylovora* was applied over *S. melonis* with no delay, we see strong growth, shown by the thick appearance of the colony (picture c), Figure

9). Additionally we see an effect on *S. melonis* outside of the colony area before the colony is removed. This is the only competitor interaction to show this effect, and is evidence that *E. amylovora* has a strong effect against *S. melonis* growth. When the *E. amylovora* colony is removed we see complete agar clearing and therefore conclude *E. amylovora* can prevent the growth of *S. melonis* (picture d), Figure 9). When application of *E. amylovora* is delayed by 48 hours, we see no growth and agar clearing giving no evidence that *E. amylovora* affects existing *S. melonis* populations. We conclude that *E. amylovora* has a strong preventative effect against *S. melonis* but no effect against existing populations. *S. melonis* applied with no delay to *E. amylovora* seeded in media shows strong growth and complete agar clearing when removed (picture c), d), Figure 8). When *S. melonis* application is delayed, we see no growth and no clearing. These results give us evidence that *S. melonis* can prevent the growth of *E. amylovora*, but does not affect existing growth.

2.4 Discussion

Zengerer et al. were able to achieve large halos with a large area of clear agar between the droplets and the surrounding growth [145]. Initial experiments made it clear that the strains used in this study did not produce the same style halos. We are unsure why this occurred, but we consider it is due to the use of TSA as growth media rather than Kings Agar B.

2.4.1 *E. amylovora* and *P. vagans*

P. vagans shows an effect in preventing *E. amylovora* growth and affecting existing populations. Given the findings of previous studies showing that pre inoculation is required for effective biocontrol, it was unexpected to see a treatment effect against *E. amylovora* [96]. Further studies investigating this finding would assist in developing an in depth understanding of the mechanisms behind the *P. vagans* treatment effect but it may be due to secreted antibiotic and siderophore compounds by *P. vagans* [108,151]. In this particular study I elected not to incubate strains for longer than 96 hours in order to maintain a similar time frame to the viability of an apple blossom as an *E. amylovora* host [48]. *P. vagans* success in preventing new populations of *E. amylovora* from growing is unsurprising given its use as a commercially available fire blight control method under the name BlightBan C9-1 [85]. *P. vagans* is considered a positive control to compare with the antagonistic effectiveness of *P. agglomerans*, *P. fluorescens*, and *S. melonis*.

The inability of *E. amylovora* to prevent or counteract growth of *P. vagans* I attribute to antibiotic production by *P. vagans* [108]. Both *E. amylovora* and *P. vagans* secrete desferrioxamine E as the primary siderophore so iron competition is not expected to be

significant in antagonism *P. vagas* antagonism towards *E. amylovora* [152–154]. While *in vitro* results are not always predictive of *in planta* results, *P. vagans* has in prior studies shown an antagonistic effect against *E. amylovora* on apple blossoms so we predict to see the same in our studies .

2.4.2 *E. amylovora* and *P. agglomerans*

The results give evidence that *P. agglomerans* is an antagonist of *E. amylovora*. As can be seen in picture f) in both Figures 4 and 5, an outline appears when strains are applied simultaneously regardless of which strain is embedded in the media. We consider this is due to a coffee ring resulting from heterogeneous surface tension in the evaporating 20 uL droplets leading to greater density of bacterial cells at the droplet edge [149,150]. The outline of the dried droplet is an area in which the applied strain is better able to compete against the agar strain, due to increased cell numbers, compared to the area within the coffee ring. We did not investigate further to identify the strain growing within the coffee ring, but we expect it is the droplet applied strain, given the numerous results we have showing the applied strain is able to form a colony when it is applied with no delay. We see some agar clearing when *P. agglomerans* is applied over media with embedded *E. amylovora* giving evidence that *P. agglomerans* can affect existing populations of *E. amylovora*. The coffee ring effect shows that at large population sizes, antagonistic strains are able to compete with *E. amylovora*, even when they are applied simultaneously. My results are in contradiction to prior findings that pre- colonisation is necessary for effective biocontrol, and suggests that antagonists applied at a high enough density can suppress *E. amylovora* growth [96].

P. agglomerans 299R has not been found to produce antibiotics, a common feature of many other *P. agglomerans* spp. including those identified as biocontrol agents of *E. amylovora*.

[103,124,155–159]. Antibiotic production is not essential for effective biocontrol and non- antibiotic producing mutants have been shown to suppress fire blight and reduce symptom development [103,122,158,159]. My results support these studies, and show that even without antibiotic production *P. agglomerans* 299R is able to inhibit growth of *E. amylovora*. It is unexpected that we see an agar clearing effect when *P. agglomerans* application is delayed onto an existing population of *E. amylovora*, and given the lack of antibiotics produced, this is attributed to T6SS activity [124]. The effects of antibiotics produced by some *Pantoea* strains can be mitigated by increased availability of amino acids found on apple blossom stigma so our results towards development of biocontrol unreliant on antibiotic production [160].

2.4.3 *E. amylovora* and *P. fluorescens*

The results of the *E. amylovora* and *P. fluorescens* double layer assay experiments suggest that the two strains are both able to limit the growth of each other, but neither can counteract pre- existing growth. These results provide evidence that the bacterial strains limit each other through; spatial pre colonisation, an overlap in nutritional requirements or through both of these mechanisms [96].

On an apple blossom, a heterogeneous environment with topological challenges as well as nutrient gradients, we anticipate *P. fluorescens* and *E. amylovora* will show competition for available nutrients, particularly iron which is limited on apple blossom stigma [65]. Both *E. amylovora* and *Pseudomonas* spp. have been shown to produce siderophores that bind extracellular iron for transport across the cellular membrane [59,154,161,162]. We predict that on apple blossoms we will see increased antagonism between *P. fluorescens* and *E. amylovora* manifesting as either segregation of the two strains, or as a decrease in the population size of one or both strains.

2.4.4 *E. amylovora* and *S. melonis*

Both *E. amylovora* and *S. melonis* are unable compete with existing growth of the opposing strain despite success in preventing growth. This suggests the two strains have an overlap in nutritional requirements or pre emptive exclusion of the agar by one strain. The results of the double layer assay investigating *S. melonis* in the solid media with *E. amylovora* applied on top with no delay were the only results to show an effect outside of the colony area. A small margin outside of the *E. amylovora* colony in which no strains grew was observable, with the surrounding *S. melonis* growth at a lower density, the closer it was to the colony. The observable margin outside of the *E. amylovora* colony *S. melonis* and may be the result of siderophore production by *E. amylovora* dispersed outside of the colony area limiting iron available for *S. melonis* [59,154,162].

On *Arabidopsis thaliana* leaves *Sphingomonas* spp. have been found to have a significant nutritional overlap with the plant pathogen *Pseudomonas syringae* [163163,164]. Bacterial antagonists with high overlap in nutrient requirements to a competing strain have been shown to have a greater antagonistic effect than strains with less nutritional overlap [163]. *Sphingomonas* spp. have been shown to have a primarily epiphytic population on *A. thaliana* leaves, so we expect a primarily epiphytic population on apple blossoms in the same blossom area as is colonised by *E. amylovora* [164]. The double layer assay provides evidence that *S. melonis* and *E. amylovora* are incompatible on solid media, suggesting that *S. melonis* will; thrive on blossoms, and exclude *E. amylovora* from colonising the stigma and style.

2.4.5 Summary

In comparison to the results obtained by [145] our results primarily showed growth inhibition or growth removal only on the area covered by the 20 uL droplet, the exception being interactions between *S. melonis* and *E amylovora* as discussed in section 2.3.4. The findings of Zengerer et al. 2018 showed growth inhibition outside the area of droplet application which I conclude is due to differing availability of growth limiting nutrients compared in the media used for these assays and reduced competition [145].

P. vagans C9-1, *P. agglomerans* 299R, *P. fluorescens* PF0-1 and *S. melonis* Fr-1 all showed antagonism against *E. amylovora* when inoculated with the pathogen simultaneously. *P. vagans* and *P. agglomerans* also exhibited a protective effect. All four strains also showed impaired growth when *E. amylovora* was applied simultaneously, but no effect to established populations of the antagonistic strains when *E. amylovora* application was delayed. These findings give support for two of my hypotheses; the four strains selected for this study will show an effect in inhibiting the growth of *E. amylovora* has been supported by my findings, and *E. amylovora* will impair the growth of antagonistic and potentially antagonistic strains when applied to surfaces, both artificial and plant. My results do not show support for the hypothesis that; on any given surface, both artificial and plant, preemptive colonization will determine antagonistic success.

The double layer assay lacks the morphology, nutritional ratios and spatial topology that define the apple blossom microenvironment. We expect that the results shown by the double layer assay will underestimate the degree of antagonism between *E. amylovora* and the competitive strains as the solid media has a greater area suitable for bacterial colonisation. In comparison the blossom is much smaller, and has fewer areas suitable for bacteria with

regard to both the availability of nutritional resources, and availability of protective microenvironments. Prior studies showed discrepancies between the *in vitro* and *in vivo* results [145,165]. We therefore predict that the antagonism we observed between *E. amylovora* and the competitor strains will not manifest in the same way, if at all, on apple blossoms.

Chapter 3 In planta investigations of antagonist effects on population growth of *E. amylovora*

3.1 Introduction

The primary site of infection for *E. amylovora* infection is the open blossom [37,137,166]. *E. amylovora* bacteria develop large epiphytic populations on the stigma, which under particular conditions develop into a pathogenic infection and blossom blight [1,38]. Blossom blight can develop into a tree wide fire blight disease, using nectarthodes as entry points into the plant circulatory systems throughout the tree [166,167]. Blossom blight in itself destroys the apple crop, infections that have migrated into the woody tissue can result in the death of the mature tree, and an orchard wide epidemic can destroy productivity for years with severe economic consequences [168,169]. Infections can enter the plant through wounds at sites other than the blossom resulting in shoot blight and rootstock blight, but as blossoms are the primary site for infection by fire blight, they are an important area of research towards prevention of fire blight [1,9,25,37]. Apple blossoms are only a viable host for the first three days after opening and the number of bacterial cells present on the stigma as well as the number that reach the hypanthium are strongly correlated to the risk of infection [47,48]. These observations make decreasing the population size of *E. amylovora* during this crucial time frame a clear target for fire blight prevention.

Fire blight antagonists as biocontrol strains have been made commercially available in several countries including New Zealand and their use as sustainable methods for protection

against fire blight continue to be studied (BlightBan C9-1 ™, BloomTime Biological ™, and BlossomBless ™) [145]. Antagonist success in protecting the apple blossom may be mediated by; nutrient competition, or through site exclusion of areas essential to *E. amylovora* infection such as the stigma, or a combination of both factors [1,38]. Previous studies have found that for effective biocontrol of *E. amylovora* by antagonists, the antagonistic strain needs to be present on the blossom before *E. amylovora*, which is a severely limiting factor for the use of biocontrol strains [137,138]. This study aims to better understand how antagonism between *E. amylovora* and antagonists is mediated to overcome their limitations, and optimise their effectiveness. The work presented in this chapter and chapter 4 will generate a comprehensive view on how antagonists and *E. amylovora* colonise apple blossoms. I aim to understand key events in the infection process of *E. amylovora* and how best to develop a robust population of antagonists that offers protection to apple blossoms, particularly apple blossom stigma, against fire blight infections.

In the detached flower assay *E. amylovora* and an antagonist are simultaneously applied to the apple blossom stigma to test antagonists of *E. amylovora* [145]. In conjunction with microscopical investigation as described in Chapter 4, the detached flower assay contributes towards understanding how bacterial populations of *E. amylovora* CFBP1430S, *P. vagans* C9-1, *P. agglomerans* 299R, and *S. melonis* Fr1 develop in the initial stages of infection. Of particular interest was how *E. amylovora* growth was affected by the presence of any of the antagonists, and whether the activity of the antagonistic strains in turn was affected by the presence of *E. amylovora*. We monitored bacterial populations for up to 96 hours after they were inoculated onto apple blossoms to compare the colonising process of *E. amylovora* with and without co-colonisation of *P. vagans, P. agglomerans* and *S. melonis*. As discussed in Chapter 1, a high degree of similarity between a pathogen and its antagonist is considered a

key feature for successful biocontrol. Our investigations aim to identify similarities in the colonising events of *E. amylovora* compared to the strains tested in this study as a means to predict success as a biocontrol agent.

Bacterial strains used for the detached flower assays were equipped with antibiotic resistance genes and constitutively expressed fluorescent proteins. Inoculated blossoms inoculated were used for both, the detached flower assay as well as for Chapter 4 assays. Antibiotic resistances of different strains were used to select for these strains after bacteria had been recovered from apple blossoms. The fluorescent proteins were used to distinguish two different strains both present on an apple blossom during microscopy as will be discussed in Chapter 4. These studies will inform our understanding of the infection progression of *E. amylovora* on apple blossoms and how to disrupt or prevent this process using antagonists.

3.2 Materials and Methods

3.2.1 Generation and properties of fluorescently labelled bacterial strains

Table 1. Relevant strains and properties

Strain	***E. amylovora* CFBP1403S**	***P. vagans* C9-1**	***P. agglomerans* 299R**	***S. melonis* Fr1**
Plasmid/ transposon	pMRE-165	pMRE- 147	pMRE- 133	::105-sYFP
Fluorescent protein	mScarlet-I	mClover3	sYFP2	sYFP2
Antibiotic resistances	Tetracycline, Kanamycin	Gentamicin, Kanamycin	Chloramphenicol, Kanamycin, Rifampicin	Gentamicin
Plasmid transposon Reference	Schlechter et al. 2018	Schlechter et al. 2018	Schlechter et al. 2018	Ledermann et al. 2014

Table 1 present the relevant bacterial strains and properties used in the detached flower assay and microscopic investigations as described in Chapters 3 and 4.

S. melonis::105-sYFP was available by Mitja Remus-Emsermann using a plasmid described by Ledermann et al. 2014 [170]. Fluorescently labelled bacterial strains of *E. amylovora, P. vagans* and *P. aggloemrans* were generated using mating experiments as follows. 5 mL overnight cultures of wild type environmental strains and plasmid bearing *Escherichia coli* S17-1 were centrifuged at 2000 *g* for 5 minutes. The supernatant was discarded, and 5 ml of 10 × PBS was used to resuspend the bacterial cells. Centrifugation and resuspension of bacteria was repeated a second time. 250 µL of both the wild type environmental strain, and the plasmid bearing *E. coli* were combined in a 1 mL Eppendorf tube and briefly vortexed. The mixed bacterial suspension was centrifuged at 2000 *g* for 5 minutes and the most of the

supernatant discarded, leaving 200 µL remaining in which the bacteria were resuspended. The entirety of the mixed bacterial suspension was pipetted onto a TSA plate as a dense bacteria suspension and allowed to dry. The bacteria were incubated at 30 °C overnight before they were harvested using a sterile loop and vortexed in 1 mL 1 × PBS to resuspend the bacteria. 10 µL, and 100 µL aliquots of the suspension were spread onto separate minimal medium 2 (MM2, 4.0 g/ L l-Asparagine (water free), 2.0 g/ L K_2HPO_4, 0.2 g/ L $MgSO_4$×7H2O, 3.0 g/ L NaCl, 10.0 g/ L Sorbitol, 7.5 g/ L agar bacteriological) agar plates supplemented with appropriate antibiotics to select against the autotrophic *E. coli* S17-1 and select in favour of environmental strains bearing the plasmid from *E. coli*. The remainder of the mixed bacterial suspension was centrifuged at 2000 *g* for 5 minutes after the MM2 plates were incubated overnight at 30 °C, colonies typically developed after 2- 3 days. Colonies were checked to see if they were fluorescing using a Dark Reader Transilluminator DR-88M (company name here). Fluorescent colonies were streaked onto a fresh MM2 plate and incubated at 30 °C until fresh colonies grew to confirm the presence of the plasmid in the environmental strain.which most of the supernatant was discarded with the remaining ~200 µL used to resuspend the bacterial cells which were then plated onto an MM2 plate supplemented with appropriate antibiotics.

3.2.2 Detached flower assay

3.2.2.1 Inoculation of blossoms

E. amylovora CFBP1430S and a competitor strain (*P. vagans* C9-1*, P. agglomerans* 299R*,* or *S. melonis* Fr1) were cultivated on TSA plates at 30° C overnight (approximately 15 hours). Fresh colonies for both *E. amylovora CFBP1430S* and the competitor strain were suspended in 1 × PBS and adjusted to OD_{600nm} of 1.0. Eppendorf racks were prepared by filling 2 mL of 10 % sucrose solution in alternating wells. Scotch tape was used to cover the filled wells and a hole pierced through with a 1000 µL pipette tip. To keep the blossoms at high relative humidity, 5 L plastic containers (Sistema, New Zealand) were equipped with 6 sheets of paper towels placed in the bottom with 50 mL of autoclaved deionised water poured over to moisten them before the boxes were sealed.

Freshly opened blossoms were collected from 2 year old Royal Gala apple trees grafted on an M9 rootstock with at least 1 cm of stem/ peduncle remaining at the base of each calyx (Figure 1). Anthers and anther filaments were removed at the base using a scalpel taking care not to damage the hypanthium or base of the style (Figure 3.1). Blossom age affects fire blight susceptibility, therefore blossoms collected for the detached flower assays were discarded if they had begun to show signs of ageing (For examples see Figure 2) [48,171]. Any blossoms that were malformed in any way were also discarded. Anthers and anther filaments were trimmed prior to inoculation rather than after incubation to increasing the speed of processing incubated blossoms. The 30 apple trees used for this study yielded approximately 40- 50 blossoms per day, with 40- 45 of these suitable for experimental use. The season in which blossoms were collected, lasted from December- early February, and overall there only

sufficient blossoms to investigate interactions between *E. amylovora* FBP1430S with *P. vagans* C9-1, *P. agglomerans* 299R and *S. melonis* Fr-1 within this project.

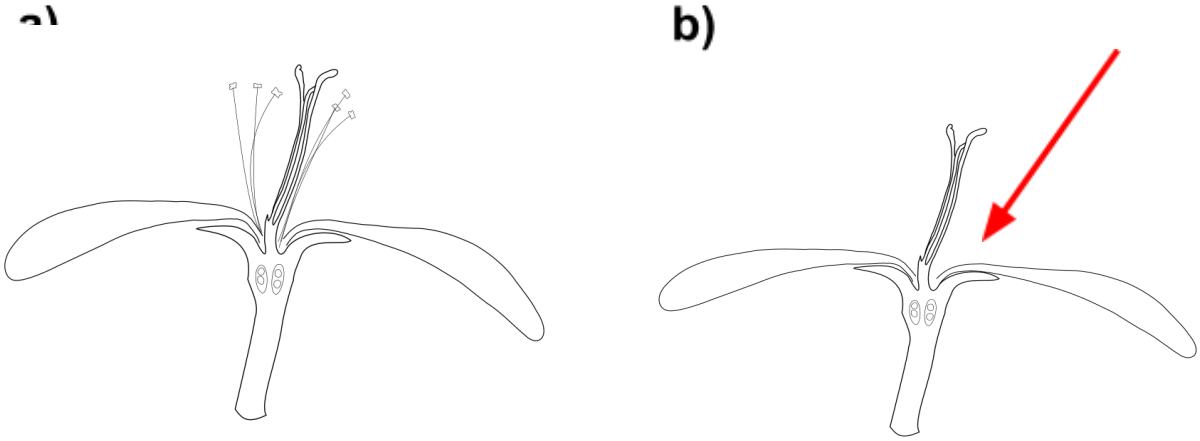

Figure 1. Blossom preparation for the detached flower assay. a) representative image of a longitudinally sliced apple blossom with all organs attached. b) representative longitudinal of a longitudinally sliced apple blossom after anthers and anther filaments have been removed.

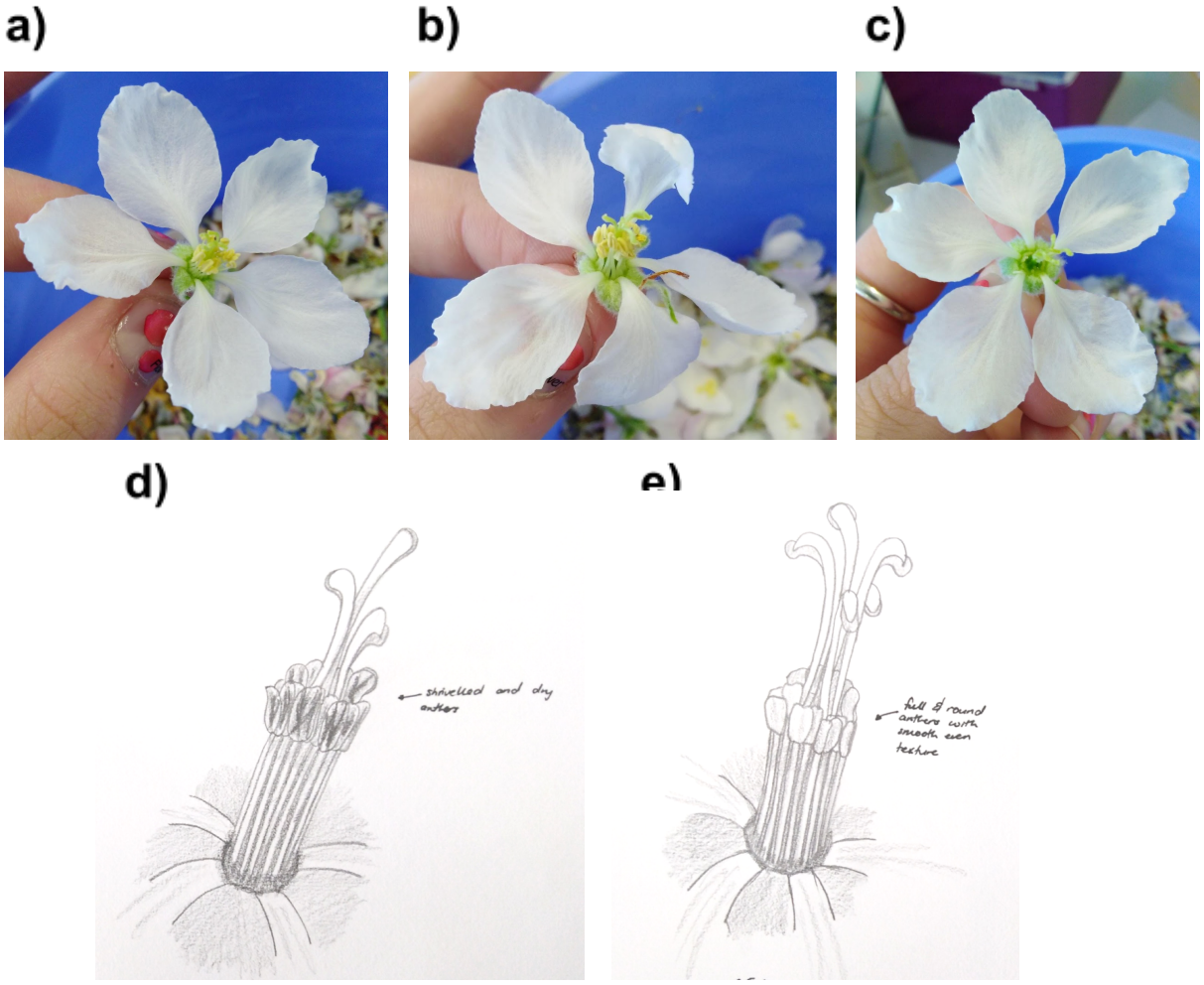

Figure 2. Identification of blossoms suitable for the detached flower assay. a) Apple blossom with signs of aging that is not suitable for use, the anthers appear drier and a darker shade of yellow. b) Apple blossom suitable for use, the anthers are plump and pale yellow. c) Apple blossom with the anthers and anther filaments removed. d) Sketch of anthers indicating blossom is not suitable for use annotated 'shrivelled and dry anthers'. e) Sketch of anthers suitable for use annotated 'full & round anthers with smooth even texture'.

The 1 x PBS *E. amylovora* suspension at OD_{600nm}= 1.0 was pipetted to fill the lid of an Eppendorf tube as shown in Figure 3. The blossom stigmas were carefully dipped into the suspension. Each stigma was dipped far enough into the suspension to just break the surface tension. Depending on the individual blossom morphology some stigmas were dipped grouped together, while others were dipped individually. 15 blossoms were inoculated with *E. amylovora*, 15 blossoms were inoculated with the competitor strain, and 15 blossoms were inoculated with a 1:1 v/v ratio of both *E. amylovora* and a competitor strain..

Figure 3. Eppendorf lid with 150 µL of resuspended bacterial culture and apple blossom ready to be dipped.

Dipping blossoms as a means of inoculation was selected in favour of pipetting. Fixing pipetted droplet onto the top of the stigma was difficult, and the droplet would not cling to the stigma well often falling down into the hypanthium making it unusable for the purpose of these experiments. Pipetting allowed an accurate count of the bacterial CFU that were applied

to each blossom, but due to the time consuming nature of this method, it was rejected. Dipping proved to be much faster and did not risk droplets falling into the hypanthium.

The blossoms were each placed into a sucrose solution filled Eppendorf rack well within the in rows of 5. The racks in turn were placed in the plastic containers with moistened paper towels and left at room temperature (approximately 21 °C) for 1 hour. After 1 hour five apple blossoms inoculated with *E. amylovora*, five inoculated with the competitor strain and five inoculated with both were sampled and the CFU of the bacterial strains were determined as explained in section 3.2.2.2. The remaining blossoms were incubated at 28 °C for 24 or 48 hours before the blossoms were sampled. A subsequent set of blossoms were prepared the following day, with 5 more blossoms inoculated for 1 hour at room temperature as well as blossoms incubated for 72 or 96 hours before the blossoms were sampled. Five blossoms were selected after each incubation period and their bacteria isolated according to the protocol in section 3.2.2.2.

3.2.2.2 Isolation of colony forming units from blossoms inoculated for 1 hour

The petals, sepals and excess stem were removed from each blossom using a scalpel as shown in Figure 4. Blossoms incubated for longer than 1 hour (24, 48, 72 and 96 hours) were sliced longitudinally from stem to stigma in one smooth motion as shown in Figure 5. As most apple blossoms had five stigma resulting in an uneven divide of stigma and style. Of the five blossoms with the same bacterial inoculation three halves were set aside for fixation as detailed in Chapter 4. The remaining blossoms had the style and hypanthium separated at the base of the style as shown in Figure 4 d), and the two sections placed into separate Eppendorf tubes filled with 1 mL of 1 × PBS each. Blossoms were always dissected in this order.

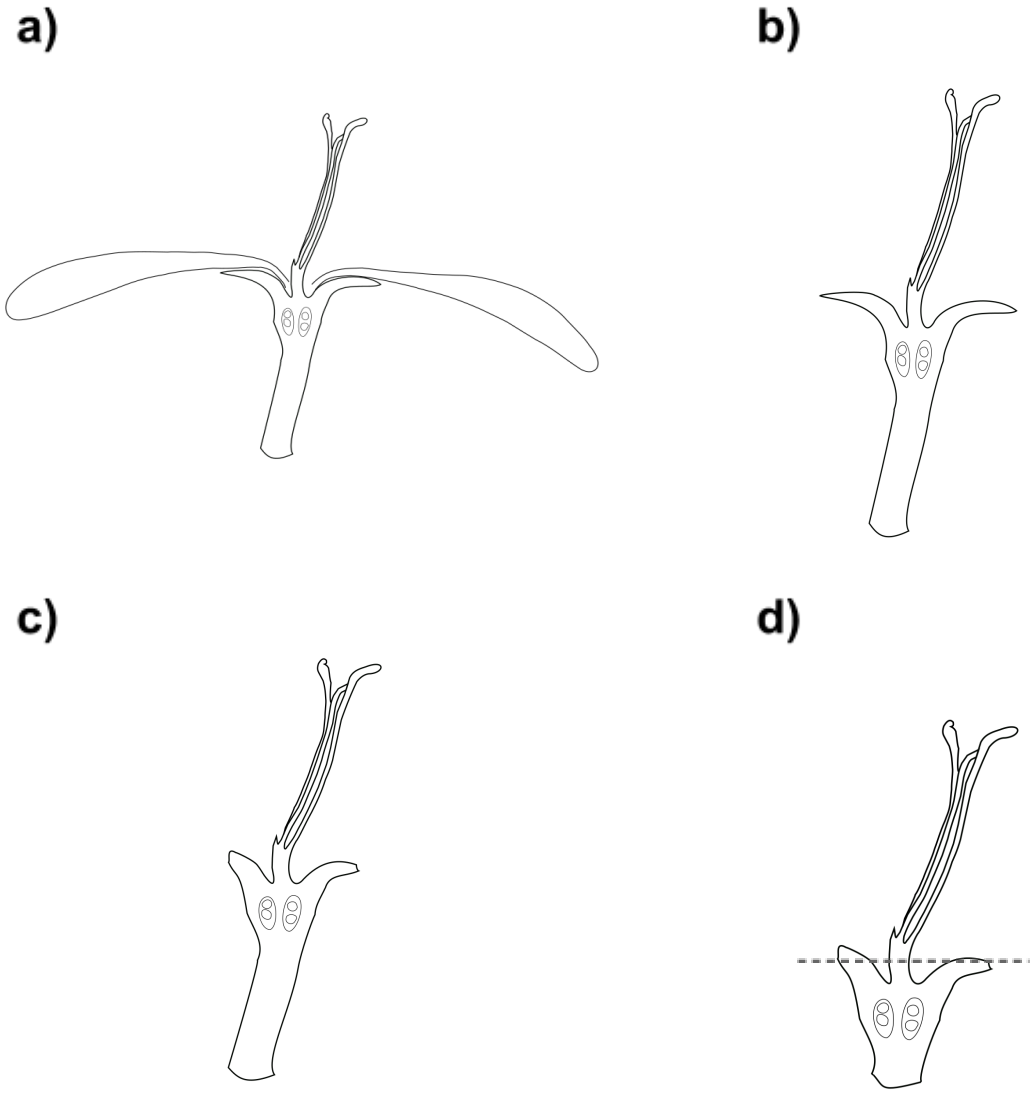

Figure 4. Preparation of apple blossoms for isolation of applied strains. a) Apple blossom with petals, sepals and stem still attached. b) Apple blossom with petals removed. c) Apple blossom with sepals trimmed. d) Apple blossom with stem trimmed with scored line to indicate where style and hypanthium are separated.

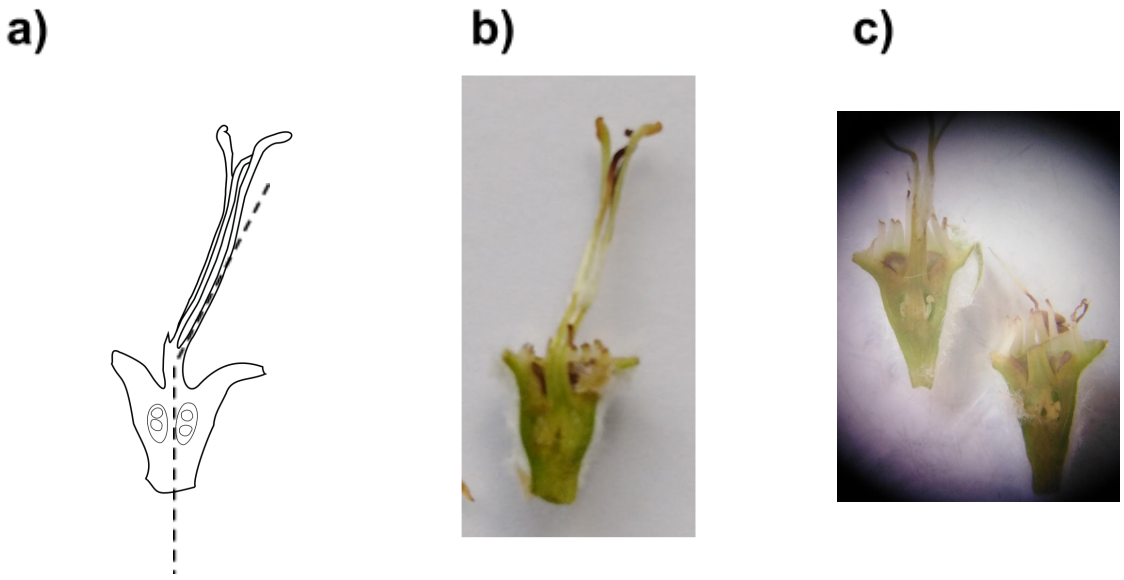

Figure 5. Examples of blossoms prepared for blossom isolation. a) Apple blossom prepared with petals, excess sepal and stem tissue removed. The scored line indicates how the blossom is sliced in half. b) An apple blossom that has been cut in half longitudinally with the stigma and style still attached. This is an example of a half apple blossom that could be used for fixation. c) Two halves of an apple blossom, one with style and stigma still attached (left), and one with the style and stigma removed (right).

Eppendorf tubes were shaken at 2.6 ms^{-1} for 2 minutes in an Omnibead rupture 24 (company and country) followed by sonication for 5 minutes. A 96 well plate was prepared with 180 µL

of PBS in rows B- H. 100 µL from each Eppendorf was pipetted into a well in row A. A multichannel pipette was used to generate a serial dilution from rows B- H.

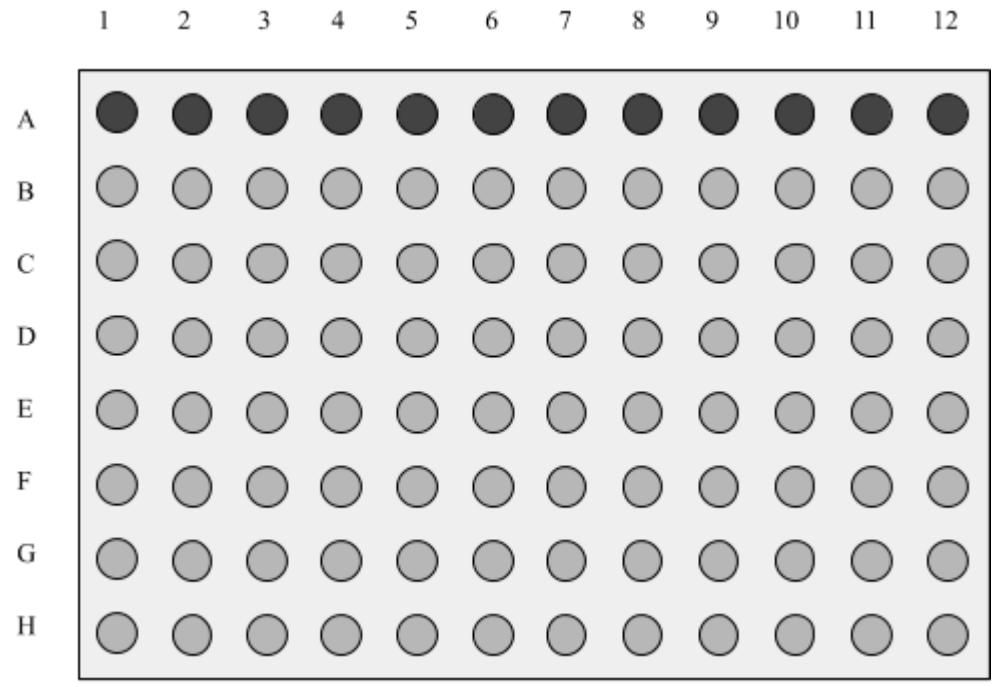

Figure 6 Representative image of a 96 well plate. The darker wells indicate where 100 µL of undiluted 1 × PBS was added.

Using a 96 pin stamp, ~3 µL of bacterial suspension was plated from every well onto TSA plates. Samples isolated from apple blossoms inoculated with two bacterial strains were plated onto two separate plates, each with appropriate antibiotics to select for one of the strains. All of the dilution wells were plated twice, sterilising the stamp in between to achieve two independent CFU counts for each well (Figure 7). All of the dilution spots were allowed to dry fully before the plates were incubated overnight or up to 48 hours at 30 °C, depending on the strain. Colonies on the plates were counted with the assistance of a stereo microscope.

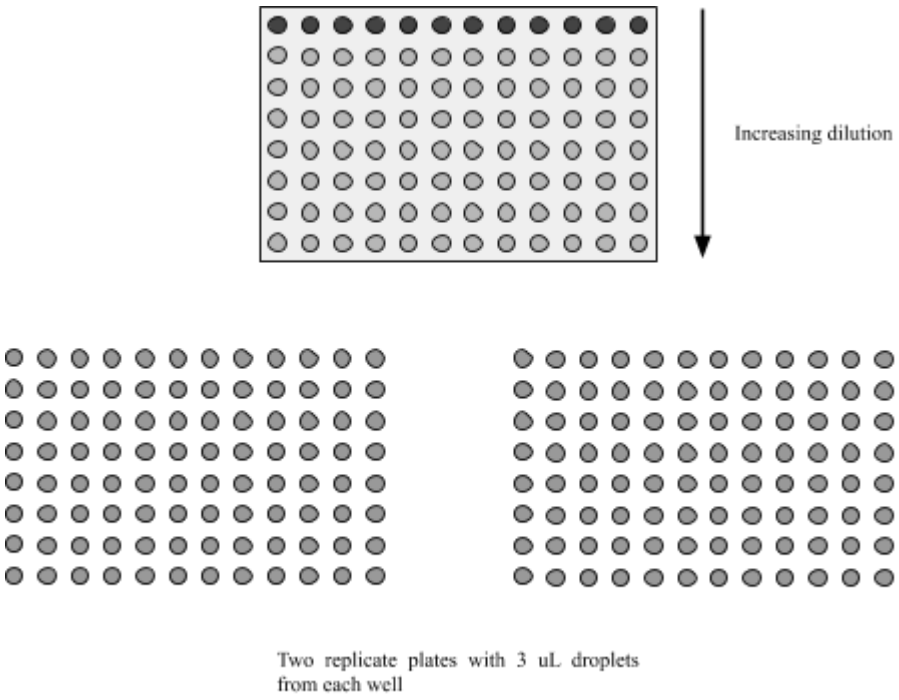

Figure 7. Representative image of a 96 well plate with diluted samples and the imprints that result on plates. Shown here are two stamps made of a single 96 well plate as were produced from diluted samples.

3.2.2.4 Data Processing and Statistical Analyses

The CFU of each dilution plated was used to calculate the bacterial CFU/ blossom in 1 × PBS for the stigma/ style and the hypanthium of each blossom. The two plates inoculated from the same 96 well plate were used to generate an average CFU/ mL.

Five apple blossoms that were all inoculated with the same treatment at the same time and incubated for the same length of time are treated as a set. Each treatment was performed twice for *P. agglomerans* and *S. melonis* investigations and once for *P. vagans* investigations. A representative set for each treatment and incubation type was selected for further analysis.

All statistical analyses were performed using GraphPad Prism 8.0.2. Collected data was transformed in the Prism programme using Y= Log (Y). A set of five blossoms was insufficient for statistical tests so normality of representative data sets is presented in the Supplementary data as graphs.

E. amylovora control CFU/ mL data for *S. melonis*, *P. agglomerans* and *P. vagans* experiments were compared to ensure the methods gave consistent results. The 1 hour, 72 hour and 96 hour incubated controls were compared using the Welch's ANOVA test.

Significant differences between the CFU of isolated *E. amylovora* were investigated using a two-way ANOVA test. The ANOVA was chosen because it allows comparison of populations with two nominal variables, in this case incubation time and inoculation treatment, and one measurement variable, in this case CFU. The two- way ANOVA method tests three hypotheses: the mean of the different populations that are incubated for different lengths of time are the same, the mean of the different populations treated with a different bacterial inoculation are the same, and there is no interaction between the two nominal variables.

3.3 Results

Limited blossom production restricted the total number of blossoms inoculated to 45 blossoms per day and limited the number of treatments that could be performed. *P. agglomerans* and *S. melonis* were inoculated individually and co inoculated with *E. amylovora* onto apple blossoms with 1, 24, 48, 72, and 96 hour incubation times. *P. agglomerans* and *S. melonis* inoculations, and co- inoculations with *E. amylovora* for all time points were performed twice, each time on five blossoms to achieve two biological replicates. Insufficient apple blossoms restricted the number of blossoms inoculated with *P. vagans* and co inoculated with *P. vagans* and *E. amylovora* to only a single set of five blossoms incubated for 1, 72, and 96 hours. *E. amylovora* inoculated apple blossoms were always prepared alongside other strains to compare the colony forming units (CFU)/ blossom inoculated to each blossom to ensure consistency of the method.

3.3.1 E. amylovora control consistency

To evaluate the reproducibility of the detached flower assay method the CFU/ blossom inoculated with a monoculture of *E. amylovora* were compared. Control treatments were performed as part of every experiment investigating the interactions of a competitor strain and *E. amylovora,* therefore they are ideal to test the robustness of the methods. The controls results are compared visually as graphs, and statistically using the Welch's ANOVA test, these results can be seen in the supplementary data. A representative set of five blossoms was selected to display on graphs as seen below (Figure 8) to show the population growth of *E. amylovora* on apple blossoms over 96 hours.

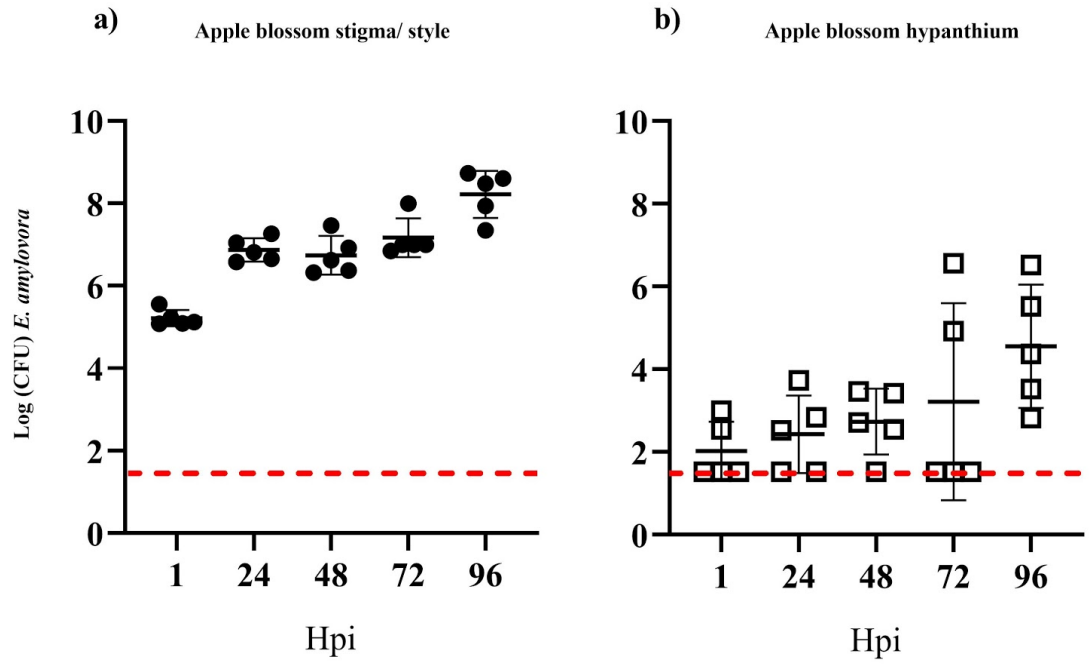

Figure 8. Growth of *E. amylovora* on apple blossoms. Each data point represents the CFU isolated from a single blossom inoculated with an *E. amylovora* monoculture. CFU were isolated from blossoms incubated for 1 hr, 24, 48, 72, or 96 hours. A representative set of 5 blossoms is presented for each incubation time. Graph a) shows CFU isolated from the stigma and style of blossoms, graph b) shows CFU isolated from the hypanthium of blossoms. The x- axis shows the hours post infection (Hpi) that blossoms were incubated for before bacteria were isolated.

Figure 8 shows representative data sets from *E. amylovora* control experiments. Blossoms were processed in replicate sets of 5 blossoms, one representative set was selected for each incubation time. The limit of detection is shown by the scored red line. CFU of *E. amylovora* CFU isolated from hypanthiums have many data points at the limit of detection and a wide data spread particularly when blossoms were incubated for longer periods of time. The data

points showing CFU isolated from the stigma and styles are more closely grouped than the CFU isolated from the hypanthium. Statistical analysis of the controls showed that there was no significant difference between the control sets performed across multiple experiments (supplementary data Table 1)

3.3.2 E. amylovora and P. vagans

The CFU of *E. amylovora* and *P. vagans* isolated from apple blossom stigma and styles after inoculation as monocultures and co- inoculations are normally distributed after being log transformed as seen in the Supplementary data shown in Figure 10 and 10. The two- way ANOVA analyses tests the following hypothesis;

> **There is no difference in the population mean of strain A inoculated onto five representative blossoms as a monoculture, compared to the population mean of strain A inoculated onto five representative blossoms inoculated as a co culture with strain B.**

This was tested with *E. amylovora* as strain A, and with *P. vagans* as strain A to investigate if co- inoculation impaired either bacteria. Comparisons of the growth of both *E. amylovora* and *P. vagans* isolated after inoculated as a monoculture and after inoculated together, from both the stigma and hypanthium are shown below in Figure 9.

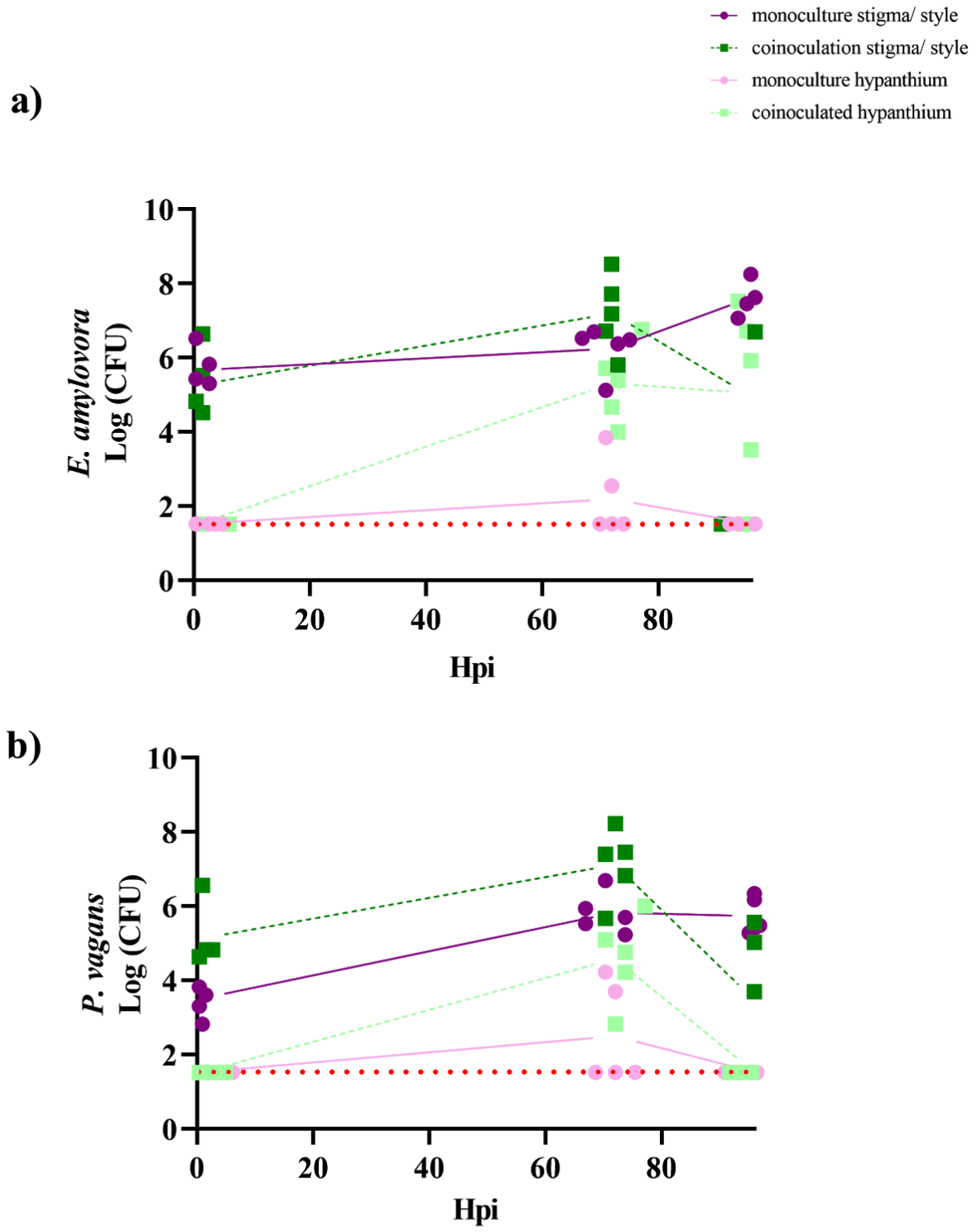

Figure 9. ***E. amylovora*** **and** ***P. vagans*** **isolated from apple blossoms. Both graphs show the CFU of bacteria isolated from five representative apple blossoms, with each data point representing one apple blossom. Graph a) compares the populations of** ***E.***

***amylovora* inoculated as a monoculture, with *E. amylovora* isolated after simultaneous inoculation with *P. vagans*. Graph b) compares the population of *P. vagans* inoculated as a monoculture, with *P. vagans* isolated after inoculation with *E. amylovora*. Solid circles plot data points from monoculture inoculated blossoms, with purple showing bacteria isolated from stigma, and pink showing bacteria isolated from the hypanthium. Square data points plot data points from co inoculated blossoms, with dark green showing bacteria isolated from stigma, and light green showing bacteria isolated from the hypanthium. The lines plot the means of the five replicate data points, with bold lines to show the monoculture inoculated blossoms, and scored lines to represent the co inoculated blossoms.**

There is increased increased variation and increased outlier presence in representative data sets isolated from both the stigma/ style and hypanthium when blossoms have been incubated for greater lengths of time which is expected to have skewed the means of some of the data sets (graphs a) and b), Figure 9). The hypanthium population of *E. amylovora* shows significant increase after 96 hours of incubation when inoculated with *P. vagans* compared to inoculation as a monoculture (graph a). Blossoms inoculated with a monoculture of *E. amylovora* show that both the stigma/ style and hypanthium populations show a slight increase after 72 hours hours of incubation compared to the isolated CFU after 1 hr (graph a), Figure 9). After 96 hours of incubation the hypanthium population of *E. amylovora* isolated after inoculation as a monoculture decreases, while the stigma and style population isolated after the same inoculation decreases. Up to 72 hours of incubation, the stigma and style populations of *E. amylovora* isolated after inoculation with *P. vagans* show a similar trend in growth compared to *E. amylovora* inoculated as a monoculture (graph a), Figure 9). After 96

hours of incubation the mean of the *E. amylovora* population isolated after inoculation with *P. vagans* decreases to the same sized that was isolated after 1 hour of incubation (graph a), Figure 9). However we do note than outliers in the data set of sigma and styles inoculated with a co- inoculation before *E. amylovora* was isolated after 96 hours may have skewed the mean of the data to be lower than expected.

In considering changes to the population of *P. vagans* when inoculated as a co- inoculation rather than a monoculture, it is important to note that the monoculture inoculated blossoms show much lower CFU after one hour of incubation, than the population inoculated with *E. amylovora*. I expect this has made the CFU isolated after greater incubation times lower than would be expected. The populations of *P. vagans* isolated after co- inoculation with *E. amylovora* show a similar trend in growth over time on both the stigma and hypanthium, with an increase between 1 hr and 72 hours, and a sudden decrease after 96 hours. The populations of *P. vagans* on stigma/ styles inoculated with *E. amylovora* show a significant decrease after 96 of incubation compared to populations inoculated as a monoculture (graph b), Figure 9). There is an outlier at the limit of detection in this data set which may have skewed the population mean. The sudden decrease after 96 hours of incubation compared to 72 hours of incubation in populations on the stigma/ style after inoculation with *E. amylovora* is also shown by populations isolated from the hypanthium (graph b), Figure 9). The stigma and style populations of *E. amylovora* and *P. vagans* both show a decrease after 96 hours of incubation compared to their respective population sizes after 72 hours when they are inoculated together (graphs a) and b), Figure 9). The hypanthium populations of *P. vagans* and *E. amylovora* isolated after inoculation together show very different dynamics, with the *P. vagans* population decreasing after 96 hours, and the *E. amylovora* population increasing slightly compared to their respective populations after 72 hours (graphs a) and b), Figure 9).

We see evidence that the stigma/ style populations of both *P. vagans* and *E. amylovora* decrease when inoculated together compared to their populations when inoculated individually. We see evidence that the *P. vagans* population on hypanthium after inoculation with *E. amylovora* is no different after 96 hours of incubation although the overall growth fluctuates compared to inoculation as a monoculture. In comparison the hypanthium population of *E. amylovora* reach much greater sizes when incubated with *P. vagans* compared to incubation as a monoculture. The two- way ANOVA test of this data found that the inoculation treatment did not affect the population sizes of *E. amylovora* or *P. vagans* on stigma and styles, but did have an affect on the hypanthium populations (supplementary data Table 2).

3.3.3 *P. agglomerans* and *E. amylovora*

The CFU of *E. amylovora* and *P. agglomerans* isolated from apple blossom stigma and styles after inoculation as monocultures and co- inoculations are normally distributed after being log transformed as seen in the Supplementary data (Figures 5 and 6). The two- way ANOVA analyses tests the following hypothesis;

> **there is no difference in the population mean of strain A inoculated onto five representative blossoms as a monoculture, compared to the population mean of strain A inoculated onto five representative blossoms inoculated as a co culture with strain B.**

This was tested with *E. amylovora* as strain A, and with *P. agglomerans* as strain A to investigate if co- inoculation impaired either bacteria. Comparisons of the growth of both *E. amylovora* and *P. agglomerans* isolated after inoculated as a monoculture and after inoculated together, from both the stigma and hypanthium are shown below in Figure 9.

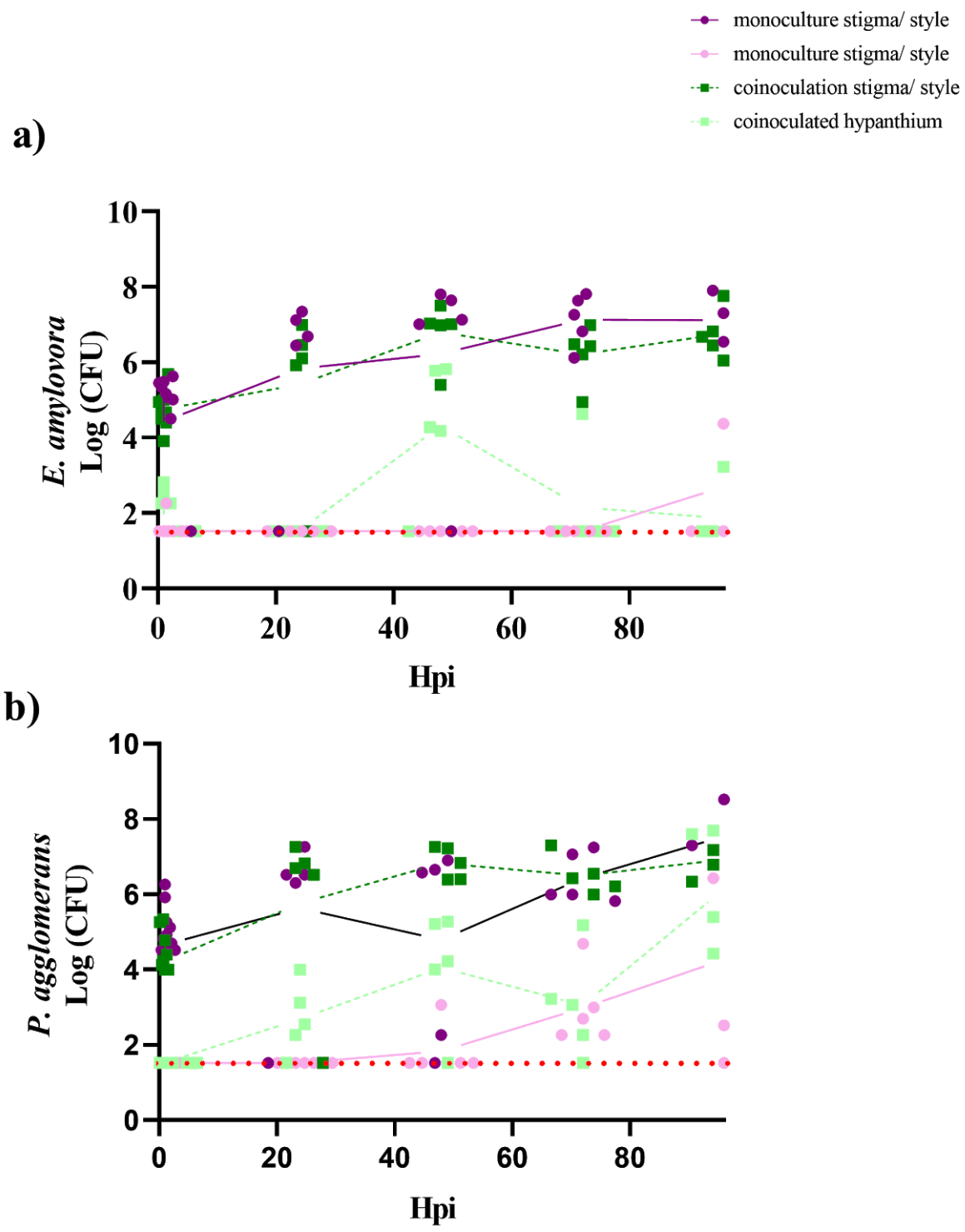

Figure 10. ***E. amylovora*** **and** ***P. agglomerans*** **isolated from apple blossoms. Both graphs show the CFU of bacteria isolated from five representative apple blossoms, with each**

data point representing one apple blossom. Graph a) compares the populations of *E. amylovora* inoculated as a monoculture, with *E. amylovora* isolated after simultaneous inoculation with *P. agglomerans*. Graph b) compares the population of *P. agglomerans* inoculated as a monoculture, with *P. agglomerans* isolated after inoculation with *E. amylovora*. Solid circles plot data points from monoculture inoculated blossoms, with purple showing bacteria isolated from stigma, and pink showing bacteria isolated from the hypanthium. Square data points plot data points from co inoculated blossoms, with dark green showing bacteria isolated from stigma, and light green showing bacteria isolated from the hypanthium. The lines plot the means of the five replicate data points, with bold lines to show the monoculture inoculated blossoms, and scored lines to represent the co inoculated blossoms.

There is no significant difference between *E. amylovora* CFU isolated from the apple blossom stigma/ style inoculated as a monoculture compared to populations inoculated with *P. agglomerans*. The CFU of *P. agglomerans* isolated from apple blossom stigma/ style after inoculation as a monoculture compared to CFU isolated after inoculation as a co culture show no significant difference. The populations of *E. amylovora* on the hypanthium after 96 hours show very little difference when inoculated as a monoculture compared to inoculation with *P. agglomerans*, although after 48 hours of incubation there is a peak at which the CFU is much higher after inoculation with *P. agglomerans* (graph a), Figure 10). These observations are supported by the two-way ANOVA test which found there was no significant difference in *E. amylovora* populations inoculated with *P. agglomerans* compared to populations inoculated as a monoculture isolated from the stigma/ style, but there is a difference found in populations isolated from the hypanthium.

The populations of *P. agglomerans* isolated from apple blossom stigma/ style show no significant differences when inoculated as a monoculture, compared to inoculation with *E. amylovora* (graph b), Figure 10). The population of *P. agglomerans* isolated from the hypanthium after inoculation as a monoculture shows a steady increase after 48 hours (graph b), Figure 10). The *P. agglomerans* populations isolated from the hypanthium after inoculation with *E. amylovora* showed a rapid increase compared to the population inoculated as a monoculture (graph b), Figure 10). The population of *P. agglomerans* on the hypanthium inoculated with *E. amylovora* began increasing after 1 hr of inoculation, and with the exception of the CFU after 72 hour of incubation, increased steadily (graph b), Figure 10). The mean after 72 hours may be a variation in the expected trend given that the means of 24, 48, and 96 hour incubated blossoms overall follow a singular trend line (graph b), Figure 10). The populations of *E. amylovora* and *P. agglomerans* inoculated together and isolated from apple blossom stigma/ style both show a similar trend in growth (graphs a) and b), Figure 10). The hypanthium populations of *E. amylovora* and *P. agglomerans* inoculated together show opposite growth trends, the *E. amylovora* population decreases after 48 hours of incubation, while the *P. agglomerans* population increases after 48 hours of incubation (graphs a) and b), Figure 10). The two- way ANOVA analysis found that inoculation together did not affect *E. amylovora* or *P. agglomerans* populations on apple blossom stigma/ style (supplementary data, table 3). However for both *E. amylovora* and *P. agglomerans*, inoculation together affected the population size that was achieved on the hypanthium.

3.3.4 *S. melonis* and *E. amylovora*

The CFU of *E. amylovora* and *S. melonis* isolated from apple blossom stigma and styles after inoculation as monocultures and co- inoculations are normally distributed after being log transformed as seen in the Supplementary data (Figures 7 and 8). The two- way ANOVA analyses tests the following hypothesis;

> **there is no difference in the population mean of strain A inoculated onto five representative blossoms as a monoculture, compared to the population mean of strain A inoculated onto five representative blossoms inoculated as a co culture with strain B.**

This was tested with *E. amylovora* as strain A, and with *S. melonis* as strain A to investigate if co- inoculation impaired either bacteria. Comparisons of the growth of both *E. amylovora* and *S. melonis* isolated after inoculated as a monoculture and after inoculated together, from both the stigma and hypanthium are shown below in Figure 9.

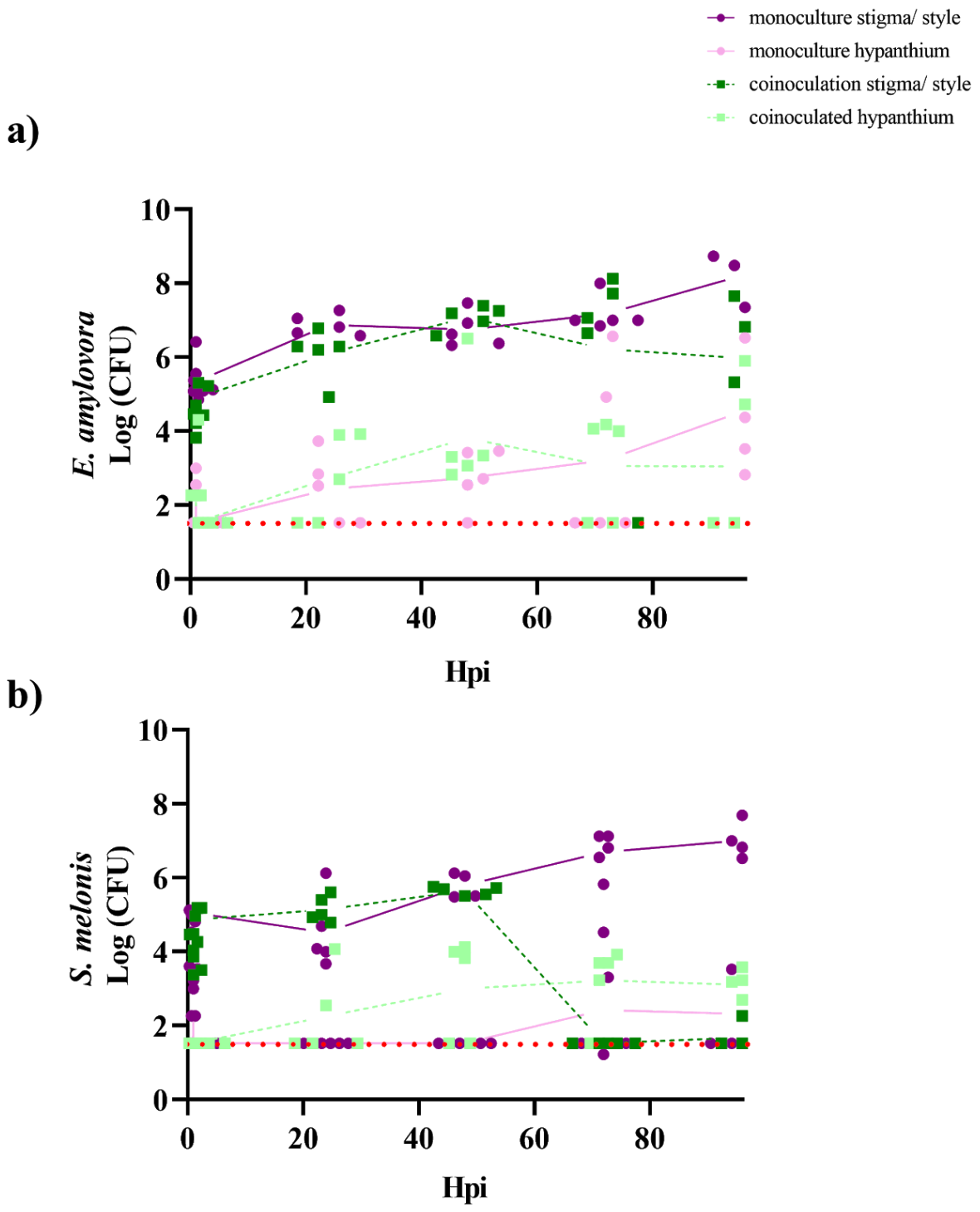

Figure 11. *E. amylovora* and *S. melonis* isolated from apple blossoms. Both graphs show the CFU of bacteria isolated from five representative apple blossoms, with each data point representing one apple blossom. Graph a) compares the populations of *E.*

***amylovora* inoculated as a monoculture, with *E. amylovora* isolated after simultaneous inoculation with *S. melonis*. Graph b) compares the population of *S. melonis* inoculated as a monoculture, with *S. melonis* isolated after inoculation with *E. amylovora*. Solid circles plot data points from monoculture inoculated blossoms, with purple showing bacteria isolated from stigma, and pink showing bacteria isolated from the hypanthium. Square data points plot data points from co inoculated blossoms, with dark green showing bacteria isolated from stigma, and light green showing bacteria isolated from the hypanthium. The lines plot the means of the five replicate data points, with bold lines to show the monoculture inoculated blossoms, and scored lines to represent the co inoculated blossoms.**

The populations of *E. amylovora* isolated from apple blossoms stigma/ style and incubated for up to 48 hours show no difference in size when inoculated with *S. melonis* compared to inoculation as a monoculture (graph a), Figure 11). After 48 hours, the population of *E. amylovora* inoculated with *S. melonis* decreases compared to the population of *E. amylovora* inoculated as a monoculture. The populations of *E. amylovora* isolated from hypanthium show little variation between those inoculated as a monoculture and those inoculated with *S. melonis*, although on blossoms inoculated for 96 hours, *E. amylovora* populations inoculated as a monoculture achieved greater population sizes than those inoculated with *S. melonis* (graph a), Figure 11).

The populations of *S. melonis* isolated from stigma/ style after 48 hours of incubation show a drastic decrease in size after 48 hours of incubation when inoculated with *E. amylovora* compared to inoculation as a monoculture (graph b), Figure 11). Populations of *S. melonis*

inoculated with *E. amylovora* and isolated from the hypanthium reach and maintain greater population sizes than populations inoculated as a monoculture (graph b), Figure 11).

The populations of *E. amylovora* and *S. melonis* isolated from the stigma/ style after inoculation together are lower than their respective monoculture inoculated population sizes (graphs a) and b), Figure 11). Despite the drastic decrease in *S. melonis* population when inoculated with *E. amylovora*, there is still an effect on the *E. amylovora* population that remains on the stigma/ style (graphs a) and b), Figure 11). The populations of *E. amylovora* and *S. melonis* isolated from the hypanthium after inoculation together follow a similar trend when isolated after up to 48 hours after inoculation (graphs a) and b), Figure 11). After 48 hours, the population of *E. amylovora* slightly decreases, while the population of *S. melonis* continues to grow in size (graphs a) and b), Figure 11). The two- way ANOVA analysis showed that *S. melonis* and *E. amylovora* population sizes isolated from the hypanthium were both affected by their inoculation together, while populations isolated from the stigma/ style were unchanged (Supplementary data, Table 4).

3.4 Discussion

3.4.1 E. amylovora control results

E. amylovora controls isolated from apple blossom stigma and styles shows a steady increase in population size with greater incubation time. The controls are consistent between multiple replicates showing that the method is robust. The control results give good confidence in the method and we expect the inoculation and bacteria harvesting methods will be consistent throughout the detached flower assays. A lot of hypanthium data points were at the limit of detection regardless of incubation time so it is expected that microscopy research of bacterial populations will yield very few or undetectable bacterial populations.

3.4.2 *E. amylovora* and *P. vagans*

There is no evidence that *E. amylovora* populations isolated from stigma/ style were affected by inoculation with *P. vagans* (Figure 10 of the Chapter 3 results section, and Supplementary data Table 2). These results support previous findings that pre colonisation is necessary for effective antagonism, as we saw no antagonism and strains were inoculated together [96]. However Zenenger et al. (2018) found that *P. vagans* C9-1 co inoculated with *E. amylovora* was able to reduce the pathogen population sizes [145]. The assays performed by Zenenger et al. (2018) were performed by directly inoculating the hypanthium rather than the stigma of apple blossoms which suggests different inoculation application yields different results [145].

Populations of *E. amylovora* and *P. vagans* isolated from the hypanthium are found to be higher than their respective monoculture inoculated blossom populations when inoculated together. Based on these findings *E. amylovora* colonisation of the hypanthium is promoted or facilitated by *P. vagans*. This is in significant contrast to studies which report *P. vagans* as

a strong antagonist of *E. amylovora* [29,85,97,98,145]. Even if co- inoculation is not sufficient for biocontrol of fire blight, an effect which favours *E. amylovora* invasion of the hypanthium is very unexpected. The results obtained do not support my hypothesis that *P. vagans* will show an effect in inhibiting the growth of *E. amylovora*, but does support the hypothesis that preemptive colonization will determine antagonistic success. When a population of *P. vagans* was inoculated with a larger population of *E. amylovora*, as can be seen by the 1 hr population sizes (graphs a) and b), Figure 9), despite known mechanisms that facilitate biocontrol, there was no limiting effect on *E. amylovora*. Microscopical investigations are expected to support these findings on stigma and style, but given the low population sizes isolated from the hypanthium, there may be very few fluorescent bacteria visible on these surfaces.

P. vagans populations on stigma/ style were found to be unaffected by *E. amylovora* co inoculated onto the stigma, while the hypanthium populations of *P. vagans* were increased when inoculated onto the stigma with *E. amylovora*. The results do not support my hypothesis that antagonistic strains will show impaired growth when applied to plant surfaces with *E. amylovora*. In fact discounting the stigma/ style data with the reduced starting inoculum, the effect of *E. amylovora* on *P. vagans* populations in the hypanthium was to increase them. These results may go towards understanding why pre colonisation of antagonists is such an important step for effective biocontrol, and the mechanisms behind which *P. vagans* biocontrol is mediated [96]. It is expected that microscopy investigations will assist our understanding of *P. vagans* and *E. amylovora* dynamics on the stigma and style. Given the difference in inoculated *P. vagans* as a monoculture and with *E. amylovora*, further investigations both microscopical and further detached flower assay are required. Given that

so few blossoms were able to be used for these assays, and small sample sizes have reduced statistical power, it is difficult to see clear trends in population growth.

3.4.3 *E. amylovora* and *P. agglomerans*

There is no evidence that *P. agglomerans* 299R and *E. amylovora* affect the population size of each other on the stigma and style when the strains are inoculated together onto the stigma. My results support previous antagonistic research which showed pre inoculation of the antagonist is necessary for successful biocontrol [96]. On the hypanthium both *E. amylovora* and *P. agglomerans* showed an increase in population size when the strains were inoculated together. These results suggest both strains are advantaged when inoculated together, potentially the result of strains increasing nutrient bioavailability to each other. If there is a mutually beneficial relationship, *P. agglomerans* 299R is unsuitable for use as a biocontrol agent, and likely has differing nutritional requirements or colonisation processes to *E. amylovora* allowing the strains to coexist [96].

3.4.4 *E. amylovora* and *S. melonis*

S. melonis and *E. amylovora* decreased the population size of each other after 48 hours on the apple blossom stigma and style. This is in contradiction to the findings by Nuclo et al. (1998) and Wilson and Lindow (1993) that pre inoculation is necessary for biocontrol success [137,172]. *S. melonis* showed a high degree of antagonism towards *E. amylovora* which I attribute to nutrient and spatial competition since *S. melonis* has not been found to secrete antagonistic compounds. In comparison to *Methylobacterium*, *Pseudomonads* have a broader substrate range and were found to exhibit greater biocontrol over the plant pathogen *P. syringae* on *A. thaliana* [173]. A broad substrate range may also facilitate *S. melonis* antagonism towards *E. amylovora*. *E. amylovora* inoculation of stigma with *S. melonis* did not show any effect on

the population size of either strain in the hypanthium. However *S. melonis* inoculated with *E. amylovora* was found to show a different growth pattern than *S. melonis* inoculated as a monoculture, and the two way ANOVA test calculated a P. value of 0.0676, which is close to the limit of significance.

3.4.5. Summary

The detached flower assay is a robust and consistent method for investigating how bacterial populations grow on apple blossoms and how these populations are affected by antagonistic strains. I consistently saw that the stigmatic bacterial populations are much greater than the hypanthium populations. *P. vagans* and *P. agglomerans* were found to have no effect on the population of *E. amylovora* on stigma and style, and on the hypanthium were found to increase the populations of *E. amylovora*. *S. melonis* was the only strain found to decrease populations of *E amylovora*, and this effect was limited to populations on the stigma and style, with no effect seen on the hypanthium. As *P. vagans* was expected to show a strong antagonistic effect, these results are surprising, and given the similarity in the effects of *P. vagans* and *P. agglomerans*, *P. agglomerans* may have antagonistic potential not detected by these assays. *S. melonis* as an antagonist merits further investigation given the effect in decreasing populations on the stigma and style. Inoculating the stigma of blossoms with sprays is an easy application method, and the ability of *S. melonis* to inhibit *E. amylovora* when strains are co inoculated suggests that pre inoculation is not required for effect antagonism by this strain, a unique feature given pre inoculation is considered a requirement for biocontrol [96].

Microscopy will give information regarding colonisation events on a heterogenous environment at single cell resolution. The detached flower assay assumes changes to

population size is a predictor of successful biocontrol. While *E. amylovora* population size is correlated with risk of infection, there are important blossom sites such as the nectarthodes that need to be accessed for development of fire blight disease [1,25,45,47]. If a population of antagonists smaller than the population of *E. amylovora* is able to prevent migration to the nectarthodes, they will still be an effective fire blight biocontrol method. As was observed in my research, *S. melonis* did not inhibit population growth on the stigma and style, but did have an effect on the hypanthium populations so may still prove to be an effective biocontrol agent. The potential of the *P. vagans*, *P. agglomerans* and *S. melonis* will be investigated further in Chapter 4.

Chapter 4 *In planta* investigation of the effect of antagonists on growth of *E. amylovora* using fluorescence microscopy

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4.1 Introduction

Fluorescent protein markers are an efficient tool for following the activity of bacteria without disrupting the plant tissue [170,174,175]. Prior studies have used fluorescent proteins as a means to track single- cell bacterial activity *in planta* [176,177]. The apple blossom is a heterogeneous environment with areas which secrete nutrients and moisture including amino acids, sugars, and iron which are utilised by bacteria [25,42,65,96]. Competition for these resources facilitates antagonism between strains which can be mediated by secretion of extracellular compounds by bacteria [96,103,178,179]. Exploiting antagonism for biocontrol of a pathogen often leads to inconsistent results and effective biocontrol is heavily dependent on pre inoculation of the antagonists which greatly affects the degree to which antagonists can mitigate fire blight [96,103,178–180]. This research will contribute towards understanding of how small stigmatic populations of *E. amylovora* develop into large populations in the hypanthium and how antagonists disrupt this process. Improving the effectiveness of biocontrol strains for fire blight management will increase the efficiency of biocontrol strain and decrease reliance on increasingly ineffective antibiotic treatments [29,73,74,181].

The colonisation of *E. amylovora* CFBP1430S, *P. vagans* C9-1, *P. agglomerans* 299R, and *S. melonis* Fr-1 on apple blossom stigma was mapped using fluorescently tagged bacteria. *E. amylovora* and antagonists were also inoculated together to assess the effect on each both

pathogen and antagonist of inoculation together. Spinelli et al. 2005 found that *P. vagans* C9-1, and *E. amylovora* both colonise the papillae cells on the stigma followed by migration down the stylar groove towards the hypanthium [177]. Strains with lower antagonism towards *E. amylovora* were found to colonise different areas of the stigma [37,177]. *P. vagans* C9-1 was used as a positive control to compared to other antagonists. Effective antagonists are expected to colonise the apple blossom stigma in the same manner as *P. vagans* and *E. amylovora*. The research undertaken in this Chapter will explore my hypothesis that; strains with an antagonistic effect against *E. amylovora* will colonise apple blossoms in the same way as the pathogen.

4.1.2 Fluorescent proteins

The spectral properties of the proteins used to label relevant strains were selected to be bright and have differing absorption and emission wavelengths in order to identify individual strains. *E. amylovora*, *P. vagans*, and *P. agglomerans* were labelled with fluorescent proteins constitutively expressed under the control of the *nptII* promoter [174]. The broad host range plasmid constructed to carry the fluorescent proteins was developed by Miller et al 2000 as a broad host range plasmid containing the pBBRI origin of replication [182]. As is shown in section 3.2.1.1 of the previous chapter, the absorption and emission spectrum of the mScarlet-I protein vary from the spectrum of fluorescent proteins borne by the antagonists allowing for differentiation of bacterial strains on the blossom surface.

S. melonis::105-sYFP was provided by Mitja Remus-Emsermann developed using a plasmid described by Ledermann et al 2014 [170]. *E. amylovora*, *P. vagans*, and *P. agglomerans* were labelled using plasmids, as Schlechter et al. (2018) reported that fluorescent intensity of cells

with plasmid-conferred fluorescence is greater than the same fluorescent protein delivered into the chromosome by a transposon delivery system [174]. As the stigma is expected to be autofluorescent, bacteria with greater fluorescent intensity were favoured (personal correspondence Mitja Remus-Emsermann).

4.2 Materials and method

Blossoms were inoculated, incubated, and prepared for microscopy processing as described in Chapter 3, section 3.2. Early blossoms were processed with stigma and style fixed attached to the hypanthium. Stigma and style fixation was found to remove some of the bacterial load so later samples had the stigma and style separated as shown in figure 3.2.2.2.1 d) and stored as unfixed samples in freeze medium at - 80 °C as described below in section 4.2.1. The hypanthium after being separated underwent fixation before being frozen and stored.

4.2.1 Fixation and blossom storage

To fix samples, they were placed into 5 mL plastic tubes with approximately 2 mL of FFA solution (615 mL/ L dH_2O, 30 mL/ L formalin (38% formalin solution), 50 mL/ L glacial acetic acid, 305 mL/ L 100% ethanol) to completely cover the samples. The tubes were placed in a vacuum chamber without lids on. Vacuum pressure was slowly increased until small air bubbles began to come out of the blossoms. Correct vacuum pressure generated small bubbles that escape in a column, with the same appearance as bubbles that escape champagne. Vacuum pressure was adjusted as needed in order to maintain the ‘champagne bubble’ appearance. Active vacuum was maintained for an hour after which the blossoms were sealed under vacuum and left overnight at room temperature. Then, the vacuum

chamber was opened and lids placed onto the 5 mL tubes. A rapid freeze container was prepared with a metal shelf resting in liquid nitrogen. A cryomould (Sakura Finetek mould (varying sizes used), Tissue-Tek® Cryomold®) was partially filled with freeze medium (Leica Tissue Freeze Medium). Three replicate samples were placed, hypanthia with cut side up, into a prepared mould and fully covered with freeze medium. The mould with blossoms was placed onto the metal shelf in the freeze container and left until fully frozen, approximately 30 seconds. Frozen blossoms were stored at - 80 °C.

4.2.2 Preparation of slides for microscopy

Hypanthium from blossoms inoculated for 96 hours were processed further for microscopy. Excess freeze medium was trimmed around the hypanthium, and fresh freeze medium was used to mount the sample to a metal stub. The hypanthium was sliced laterally into 12 um slices using a deli-slicer before being placed onto glass slides. The embedding medium often provided enough moisture that a cover slip could be placed immediately over the sample, if it was not sufficient ddH_20 was added using a dropper before the cover slip was added.

Microscopy slides of the stigma and style were prepared by defrosting the embedded medium block and placing the stigma and style onto glass slides with ddH_20 before covering with a cover slip. Stigma samples that underwent fixation were detached from the hypanthium using a scalpel before being placed on the slide.

4.2.3 Microscopy and image processing

Fluorescence microscopy was performed on a Zeiss AxioImager.M1 fluorescent widefield microscope equipped with Zeiss filter sets 38HE, 43HE, 46HE (BP 470/40-FT 495-BP 525/50, BP 550/25-FT 570-BP 605/70, and BP 500/25-FT 515-BP 535/30, respectively), an

Axiocam 506 and the software Zeiss Zen 2.3. The different fluorescently labelled bacteria were detected using a sequential scanning mode, to take standard snap images, tile images and z- stack images. Tile images were taken with a 10% overlap between images which were stitched together using the Zen software, using the stigma fluorescence to determine the exposure time for all of the images [183]. Z- stack images were generated using the 20 × and 10 × objective lenses with an interval of 1.43 µm and 3.98 µm respectively between slices. All image analysis and processing were carried out in ImageJ/FIJI [148].

4.3 Microscopy Results

Representative images were selected to be presented in this section. The stigma and style have been shown from a variety of perspectives. The underside of the style and stigma refers to the area under the natural curvature of the style including the papillae cells that are located on this side of the stigma. The upper side refers to are area above the curve of the style. Plant cells on the stigma that are not pollen receptive papillae cells are referred to as stigma cells, while plant cells on the style are referred to as style cells.

4.3.1 *E. amylovora* inoculated onto stigma and style as a monoculture

This section presents microscopy results of *E. amylovora* inoculated onto apple blossoms as a monoculture. Apple blossom stigma and styles were examined 24, 48, 72, and 96 hours after inoculation. Apple blossom hypanthium were only investigated after incubation for 96 hours.

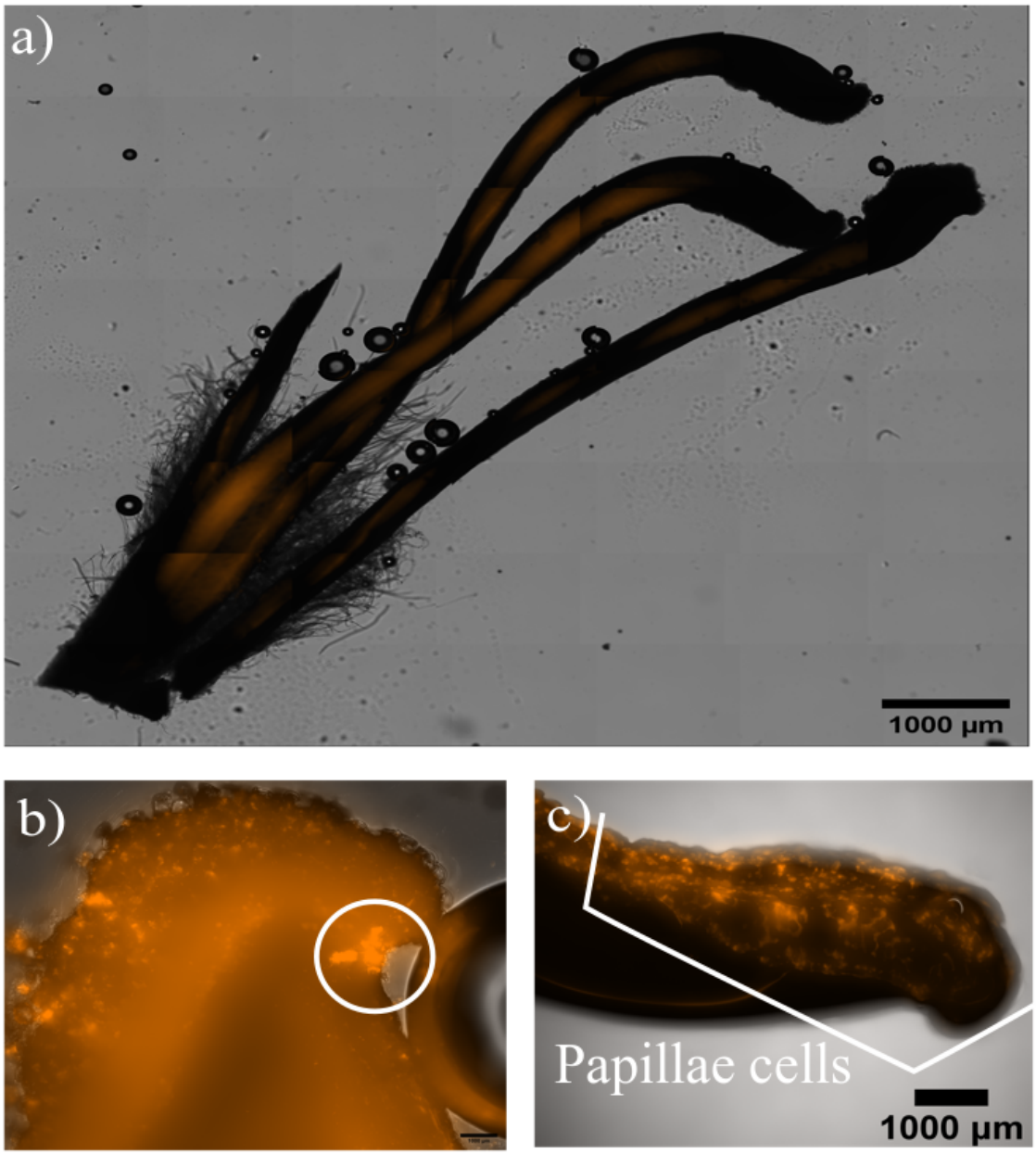

Figure 1. *E. amylovora* on stigma and styles than have been incubated for 24 hours. a) an overview of the stigma and style. b) the tip of the stigma with fluorescent *E. amylovora* circled. c) The stigma observed from a profile perspective.

After 24 hours of incubation populations of incubated *E. amylovora* on the stigma and style show little growth. There is fluorescence on the papillae cells, as indicated by images b) and c) indicating the bacteria is present exclusively on these cells, but the intensity is not very

high suggesting small populations (Figure 1). As can be seen in image a) the stigma and style are autofluorescent which decreases the contrast between the small bacterial populations and the plant cells.

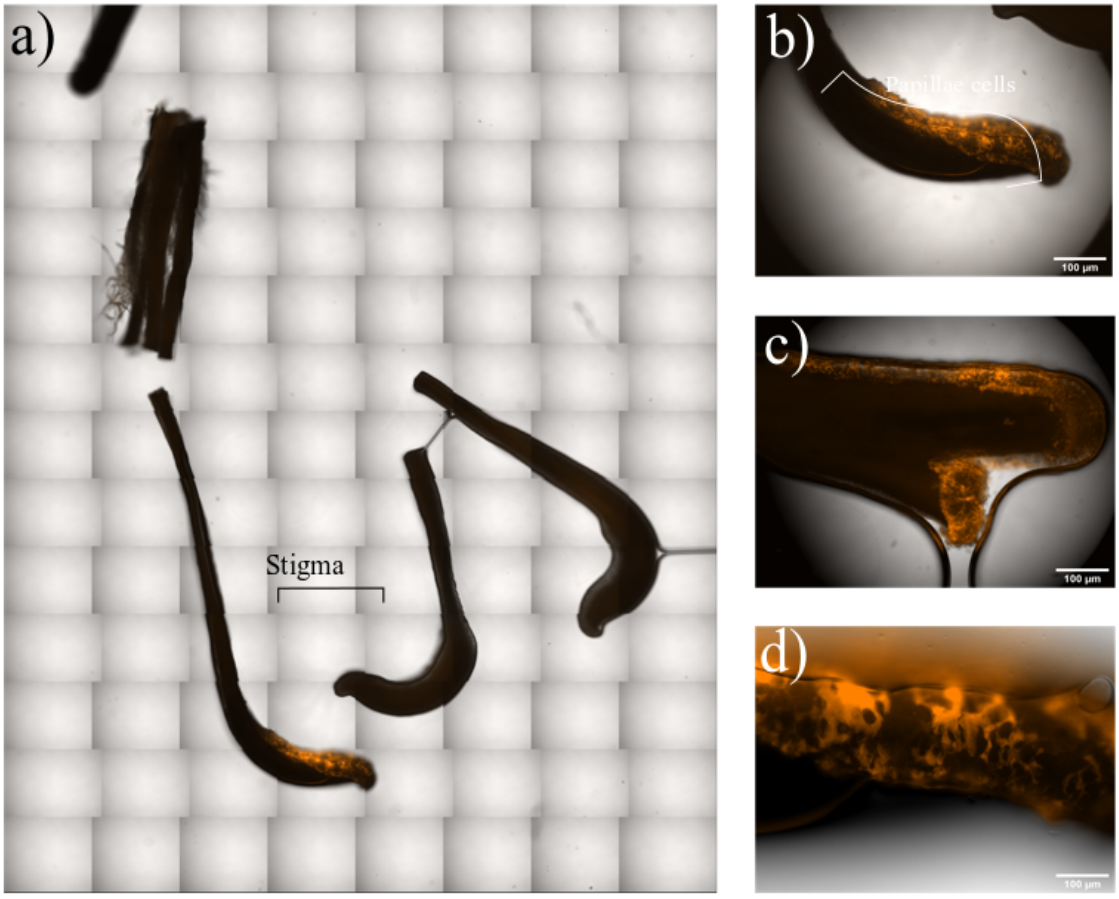

Figure 2. *E. amylovora* on apple blossom stigma after incubation for 48 hours. These samples were not fixed prior to freezing. a) Stigma and style overview. b) shows an apple blossoms stigma from a profile perspective. c) The stigma from a profile perspective. d) *E. agglomerans* aggregated in between the papillae cells.

Only one of the stigmas in image a) shows any fluorescence, suggesting colonisation of the other stigmas by *E. amylovora* is limited. As not all stigma are fluorescent, this is evidence that observed fluorescence is due to the presence of *E. amylovora* rather than autofluorescence of the plant tissue. The papillae show greater fluorescence than other areas of the blossom stigma and style although single bacterial cells are observable on the stigma and style cells as seen in images b), c), and d) (Figure 2).

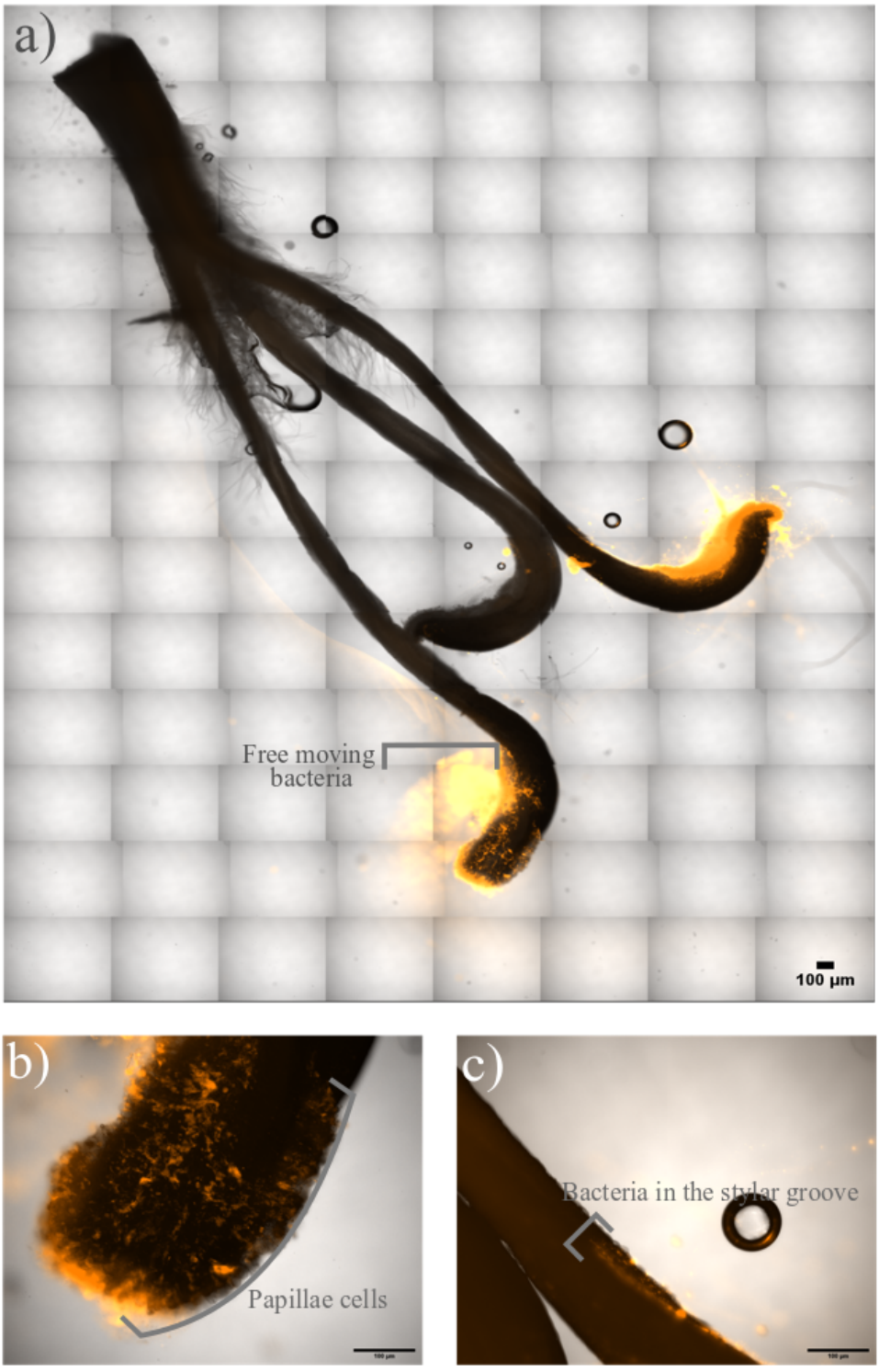

Figure 3. Populations of *E. amylovora* after 72 hours of incubation. a) Over view of the stigma and style. b) the underside of the stigma. c) the stylar groove colonised by *E. amylovora*.

There is a high fluorescence intensity indicating a large population of *E. amylovora* on the papillae cells as seen in Figure 2 a) and d). Bacterial cells on samples that were not fixed have free moving bacteria coming off them into the ddH_20 water used to prepare slides as is seen in Figure 3 a). *E. amylovora* after 72 hours of incubation has begun to colonise the apple blossom stigma, particularly the stylar groove as shown in images c) (Figure 3). There are populations on the style cells outside of the groove area as seen in Figure 3 a). The papillae cells host the most intensively fluorescent *E. amylovora* populationsas seen in Figure 3, a) and b), with *E. amylovora* cells nestled in between the papillae cells. Papillae cells with inoculated bacteria incubated for 72 hours show greater fluorescence compared to samples incubated for 24, and 48 hours.

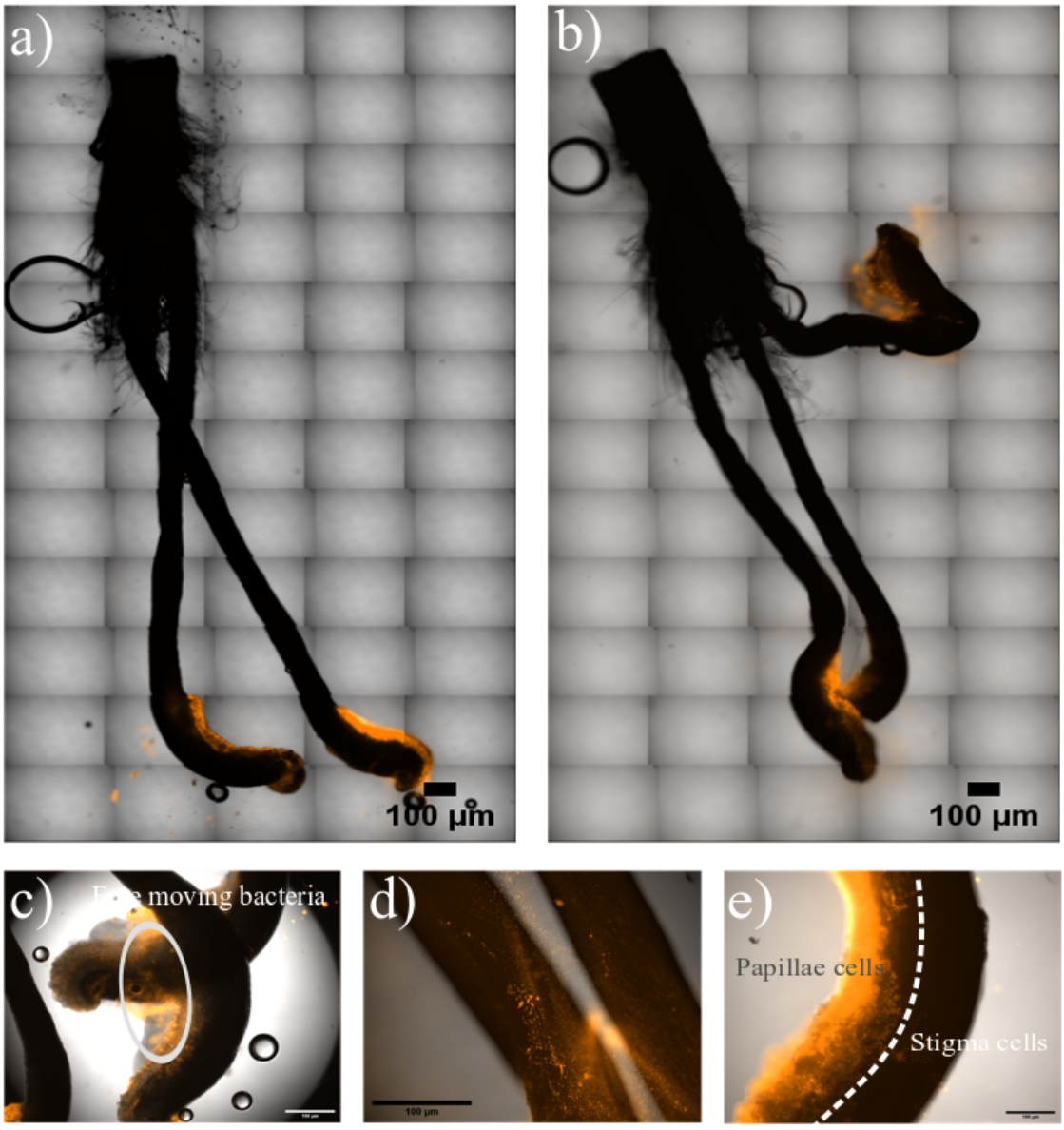

Figure 4. *E. amylovora* after incubation for 96 hours a) and b) are overviews of the stigma and style. c) two stigma with *E. amylovora* dispersing into the ddH_20 water used to prepare slides. d) Two styles, both colonised by *E. amylovora*. e) The stigma shown from a profile perspective.

After 96 hours of incubation the populations of *E. amylovora* on the papillae cells are very fluorescent indicating large population sizes (Figure 4). Bacterial cells begin to passively

diffuse into the ddH_20 water used to prepare slides as seen in images c) and d) Figure 4. *E. amylovora* cells are present on the style as seen in image d) suggesting *E. amylovora* are migrating towards the hypanthium.

The progression of *E. amylovora* colonisation and growth between 24 and 96 hours of incubation shows increasing population size particularly on the papillae cells. The stigma cells did host bacterial cells but these were at lower densities. After 72 hours of incubation, *E. amylovora* bacteria began migrating down the style towards the hypanthium.

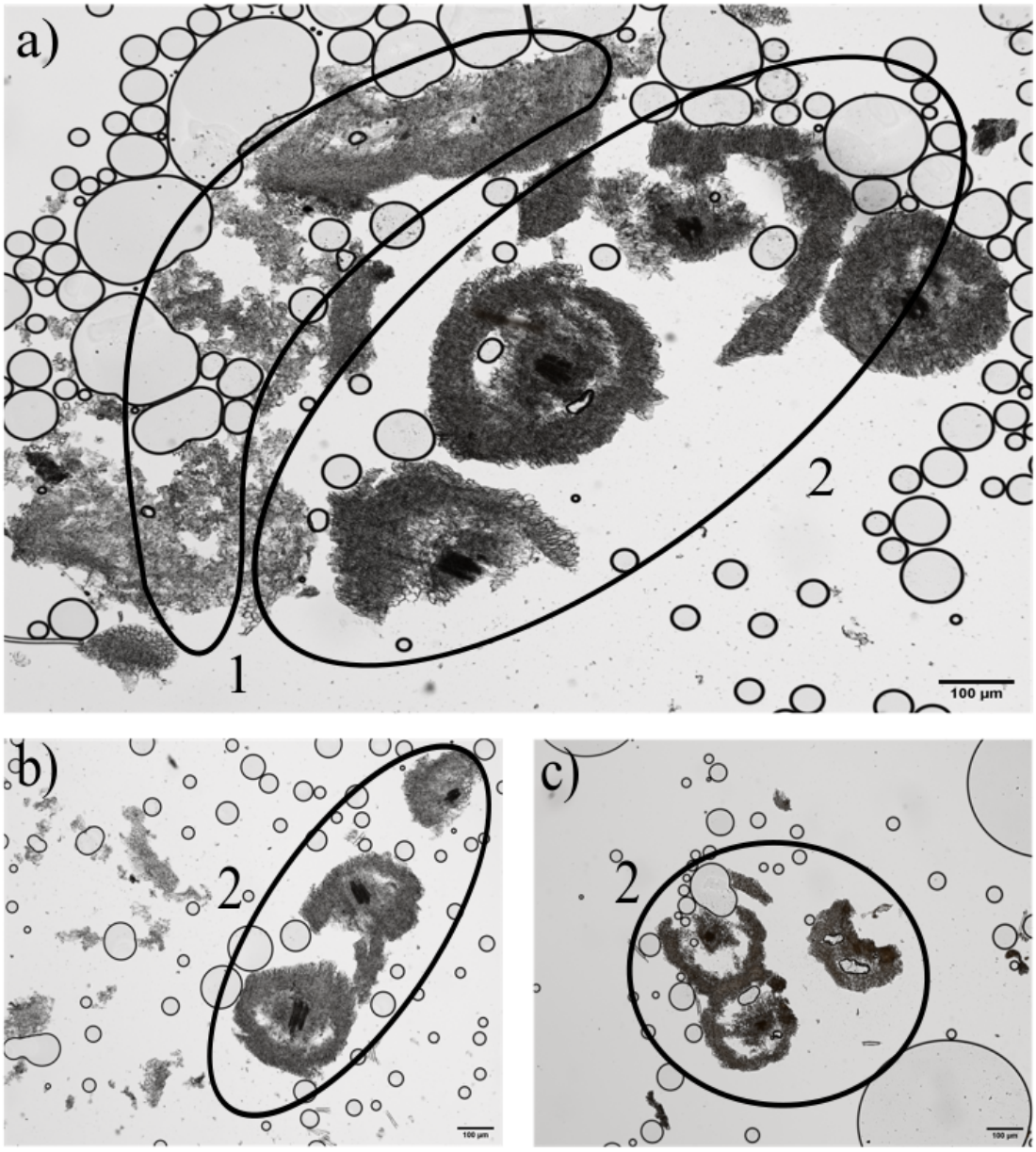

Figure 5. Hypanthium of blossoms incubated with *E. amylovora*. a) b) c) and d) all show latitudinal slices through the blossom hypanthium. 1 indicates the nectarthode cells lining the hypanthium, 2 indicates anther filaments.

There are no observable bacterial cells on the hypanthium as shown in Figure 5. The nectarthode cells are slightly darker than the other hypanthium cells, but as no bacteria are

observed it is unclear whether this can be attributed to *E. amylovora* invasion of the hypanthium, or is a natural part of blossom ageing.

In summary *E. amylovora* colonisation of the stigma and style began with growth on the papillae cells of the stigma. Greater incubation time showed increasing fluorescence intensity between the papillae cells which indicates increasing density of bacterial populations. Individual cells were observed on the stigma cells, and after 72 hours of incubation *E. amylovora* was observed on the style. Based on the results of the detached flower assay, the hypanthium did host populations of *E. amylovora* after 96 hours of incubation although no *E. amylovora* were observed in microscopy investigations. The discrepancy is considered a consequence of the fixation process which likely washed away bacterial cells.

4.3.2. Interactions of *P. vagans* and *E. amylovora* on the apple blossom

This section presents microscopy results demonstrating changes to apple blossom colonisation by *E. amylovora* and *P. vagans* populations when the strains are inoculated together. Section 4.3.2.1 shows the results of apple blossom stigma and styles inoculated with *P. vagans* and incubated for 24, 48, 72, and 96 hours as well as the apple blossom hypanthium after incubation for 96 hours. Section 4.3.2.2 shows the results of apple blossom stigma and styles inoculated with *P. vagans* and *E. amylovora* and incubated for 24, 48, 72, and 96 hours as well as the apple blossom hypanthium after incubation for 96 hours.

4.3.2.1 Results of apple blossoms incubated with a *P. vagans* monoculture

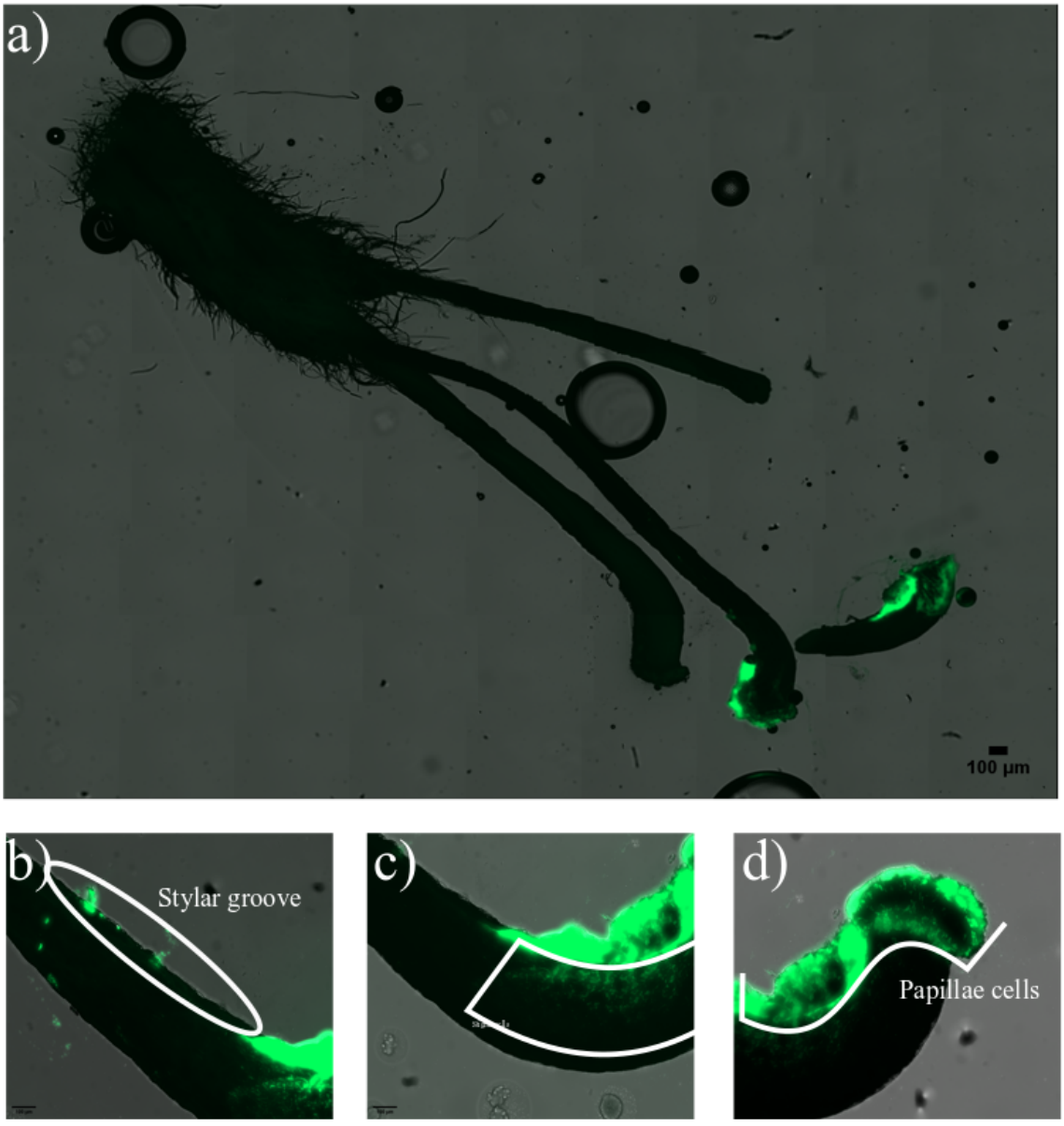

Figure 6. *P. vagans* on the stigma and style after incubation for 72 hours. a) an overview of the stigma and style. b) *P. vagans* in the stylar groove of the style. c) *P. vagans* on the stigma cells of the stigma. d) *P. vagans* on the papillae cells of the stigma.

P. vagans is observed on the papillae cells of the stigma and in the stylar groove after 72 hours of incubation as shown in image b) (Figure 6). Populations of *P. vagans* on the papillae cells are the most intensely fluorescent compared to populations on the stigma and style

suggesting higher densities of bacterial cells (Figure 6). Images a) shows that not all stigma are heavily colonised by *P. vagans*.

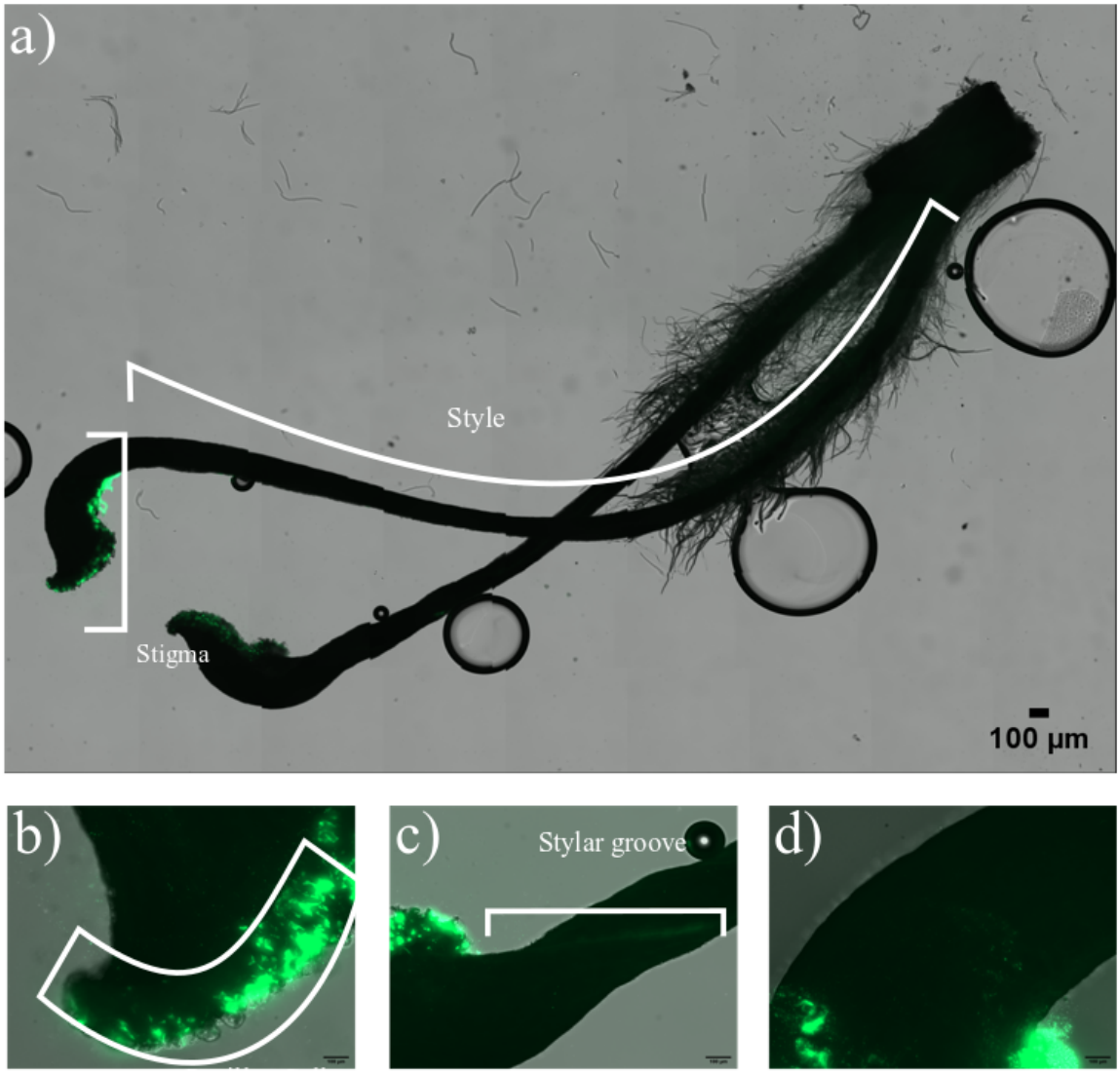

Figure 7. Apple blossom stigma and style incubated for 96 hours after inoculation with *P. vagans*. a) shows an overview of the stigma and style. b) shows the end of the stigma. c) The edge of the papillae cells and style cells with the stylar groove. d) the style from the upper side.

Some stigmas show greater fluorescence than others as shown by image a) in Figures 7. Individual *P. vagans* cells are observed in the cell margins of stigma cells.

Overall fluorescence intensity is much higher on the papillae cells than on the stigma cell margins hosting individual cells. This suggests a greater population size is hosted by the papillae cells than other areas of the style and stigma. *P. vagans* colonisation of the stigma and style is similar to *E. amylovora* in that the papillae cells host the most fluorescent populations, and the stylar groove is utilised for migration towards the hypanthium.

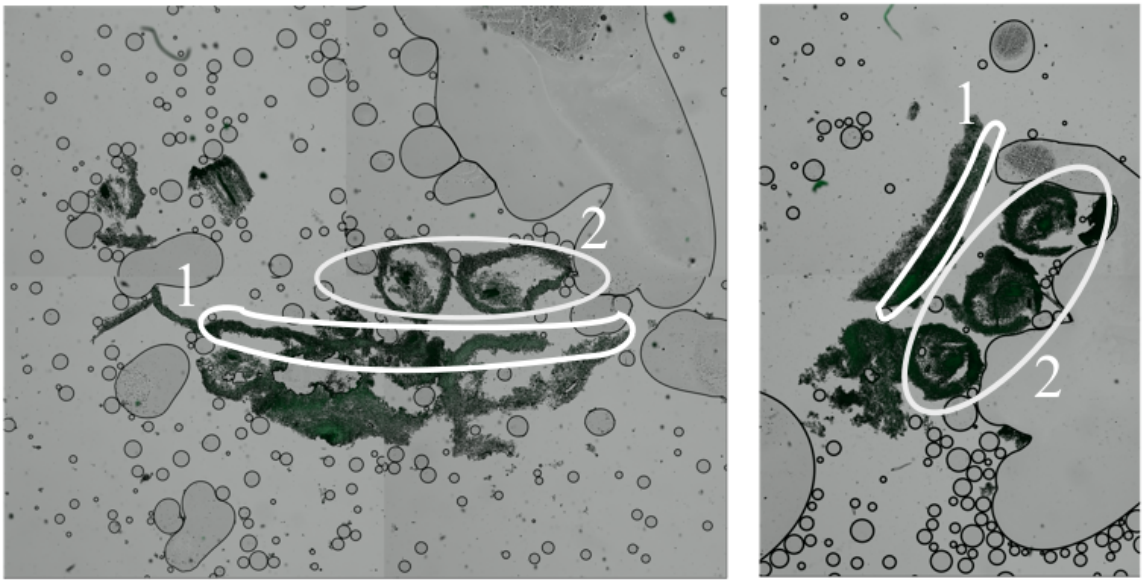

Figure 8. Hypanthium of blossoms inoculated with *P. vagans* monoculture. 1 indicates the nectarthode cells of the hypanthium, 2 indicates the anther filaments.

No *P. vagans* was observed on the hypanthium after 96 hours of incubation as shown in Figure 8.

4.3.2.2 Results of apple blossoms incubated with a *P. vagans* and *E. amylovora* co inoculation

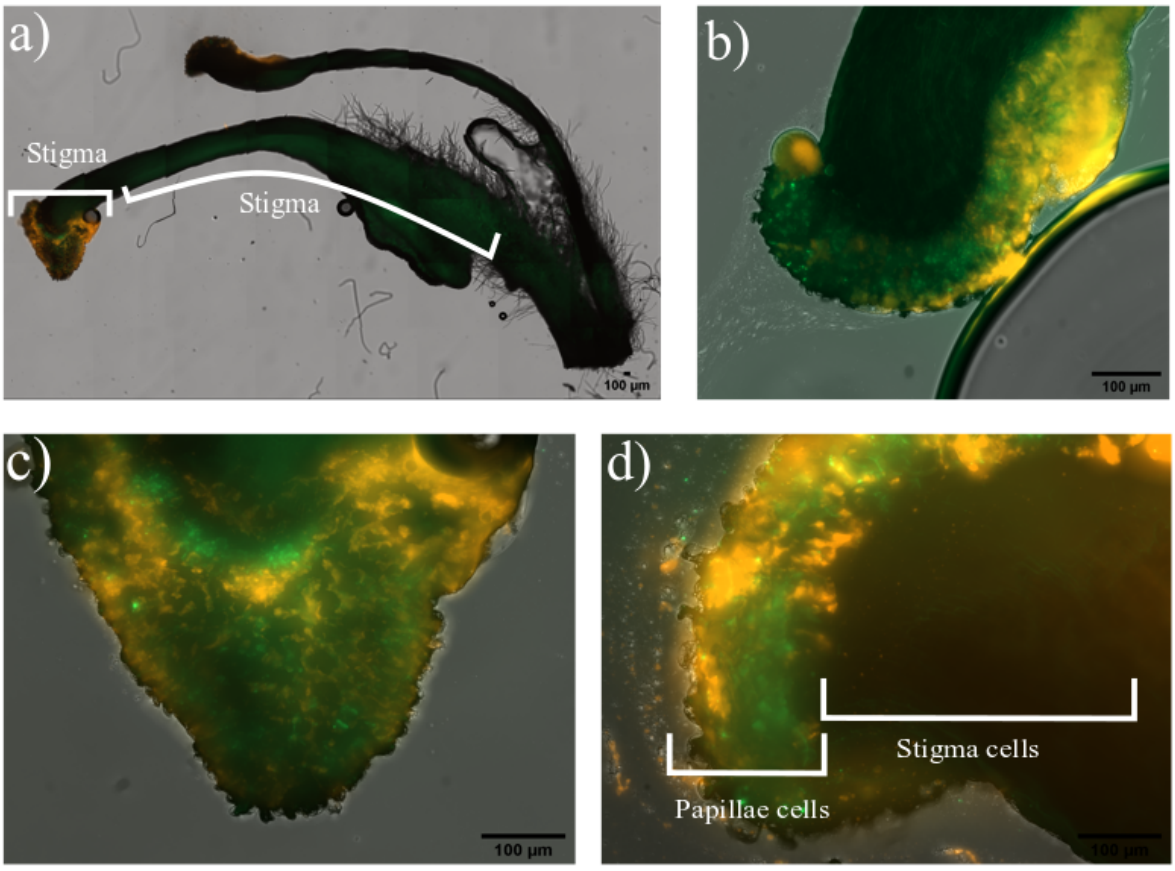

Figure 9. *P. vagans* and *E. amylovora* on the apple blossom stigma and style incubated for 72 hours after inoculation together. Images a) is an overview of the stigma and style. b) shows the tip of the stigma and motile bacteria coming off the papillae cells. c) shows the tip of the stigmas from the upper edge of the style. d) shows the stigma and style cells on the upper side of the style.

P. vagans and *E. amylovora* both grow to high densities between the papillae cells of the stigma (Figure 9). Figures c) and d) show that the edge of the papillae cells are lined with *P. vagans*, with dense populations of *E. amylovora* on the underside of the stigma. There

appears to be no change in *E. amylovora* density associated with co inoculation with *P. vagans*.

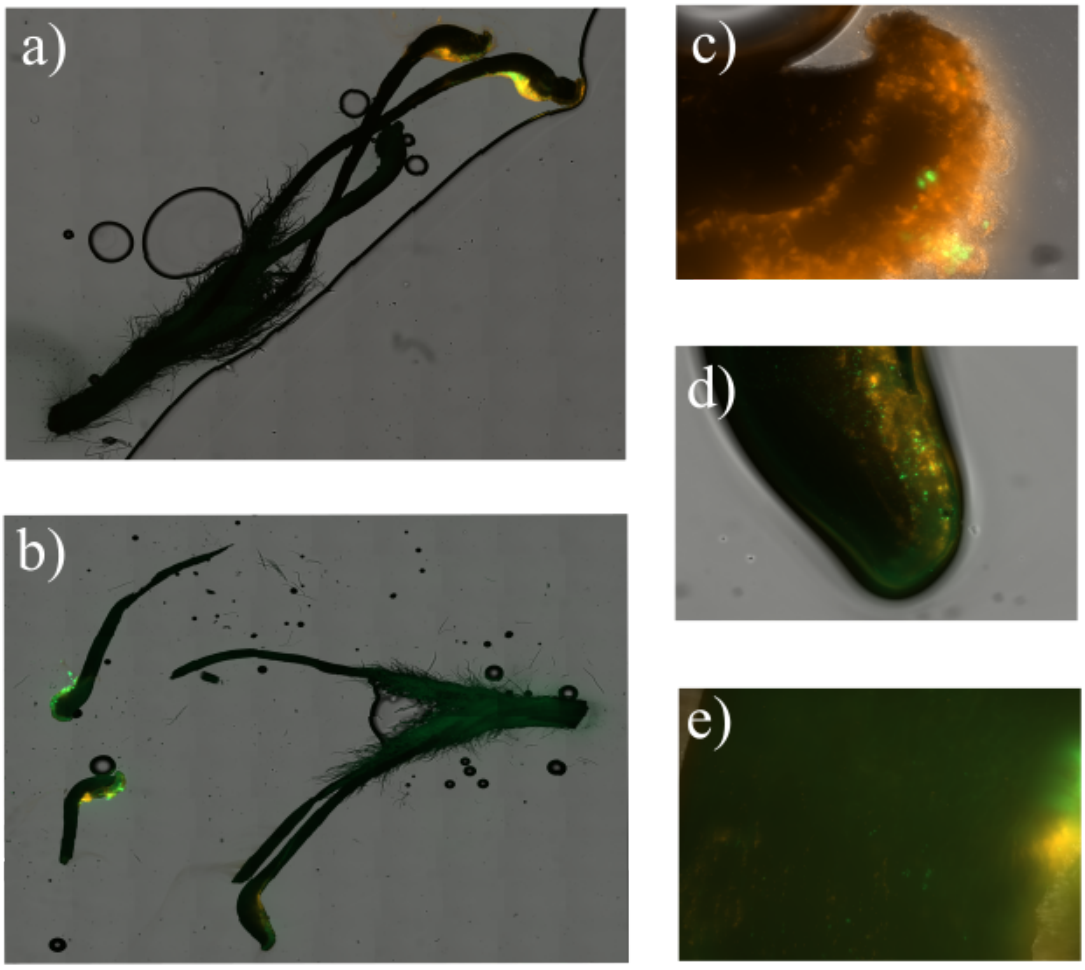

Figure 10. *P. vagans* and *E. amylovora* on the apple blossom stigma and style incubated for 96 hours after inoculation together. Images a) and b) were taken using the tile function. Images c) shows *P. vagans* and *E. amylovora* on the papillae cells. d) shows *P. vagans* on the papillae cells and adjacent stigmatic cells. e) shows *P. vagans* and *E. amylovora* cells in the cell margins of stigmatic cells adjacent to the papillae cells.

In comparison to growth of *P. vagans* and *E. amylovora* after 72 hours of incubation, after 96 hours, papillae cells of stigma are dominated primarily by a single strain as shown in Figure

10 a) and b). The papillae cells of stigma shown in in Figure 10 a) are mostly dominated by *E. amylovora*, while the papillae cells of stigma shown in Figure 10 b) are mostly dominated by *P. vagans*. In comparison to growth as a monoculture *E. amylovora* inoculated with *P. vagans* was observed less often on the style, indicating *P. vagans* inhibits migration towards the style.

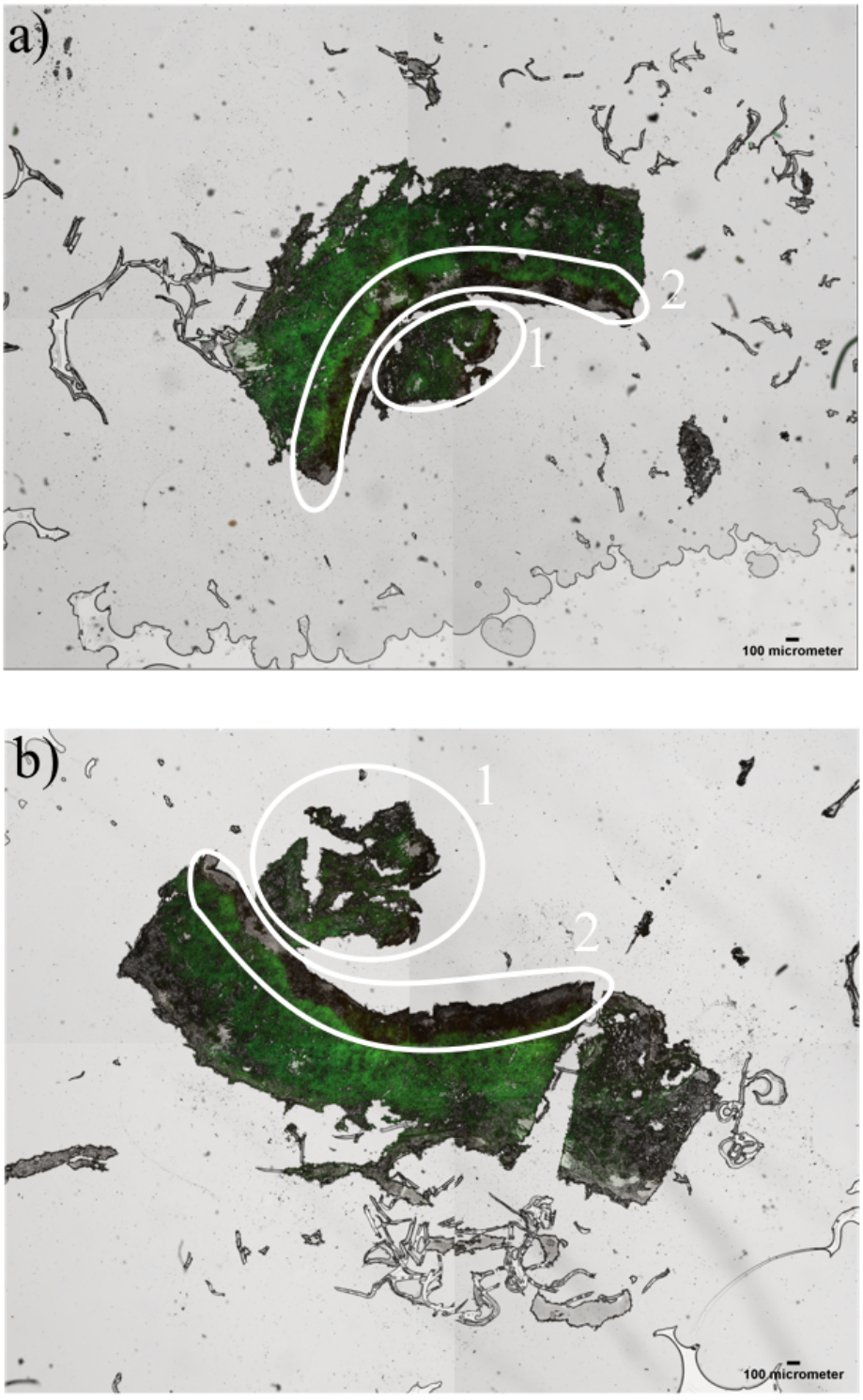

Figure 11. Hypanthium inoculated with a co inoculation of *P. vagans* and *E. amylovora*. 1 indicates apple blossom style, 2 indicated nectarthodes in the hypanthium.

There are no observable bacteria on the hypanthium and the nectarthode cells have a darker colouration than the other hypanthium cells (Figure 11). As there are no bacteria observed, this can not be attributed to a microbial effect.

In summary *P. vagans* was found to colonise the stigma in much the same way as *E. amylovora*, with large populations primarily between the papillae cells after 72 hours of incubation. The population sizes of *P. vagans* extended over less of the stigma, compared to populations of *E. amylovora* after the same incubation time suggesting growth of *P. vagans* is slower. *P. vagans* populations inoculated as a monoculture showed very little colonisation of the style, although individual cells were present between stigma cells. *P. vagans* and *E. amylovora* when colonised together were both present primarily between the papillae cells of the stigma after 72 hours of incubation with *P. vagans* growth primarily at the edge of the papillae cells, while *E. amylovora* growth was mostly in the centre. After 96 hours of incubation, distribution of *P. vagans* and *E. amylovora* was not at homogenous between different stigma. Neither *E. amylovora* nor *P. vagans* consistently dominated the papillae cells after 96 hours. As no bacteria were observed on the hypanthium after 96 hours of incubation, no conclusions can be made regarding interactions of *P. vagans* and *E. amylovora* on the nactarthode cells. *P. vagans* and *E. amylovora* reached larger population sizes with greater incubation time as shown by the detached flower assay (Chapter 3) therefore as no bacteria were present after this length of incubation, observations of samples incubated for less time were considered unnecessary.

4.3.3 Interactions of *P. agglomerans* and *E. amylovora* on the apple blossom

This section presents microscopy results demonstrating changes to apple blossom colonisation by *E. amylovora* and *P. agglomerans* populations when the strains are inoculated together. Section 4.3.3.1 shows the results of apple blossom stigma and styles inoculated with *P. agglomerans* and incubated for 24, 48, 72, and 96 hours as well as the apple blossom hypanthium after incubation for 96 hours. Section 4.3.3.2 shows the results of apple blossom stigma and styles inoculated with *P. agglomerans* and *E. amylovora* and incubated for 24, 48, 72, and 96 hours as well as the apple blossom hypanthium after incubation for 96 hours.

4.3.3.1 *P. agglomerans* inoculated as a monoculture

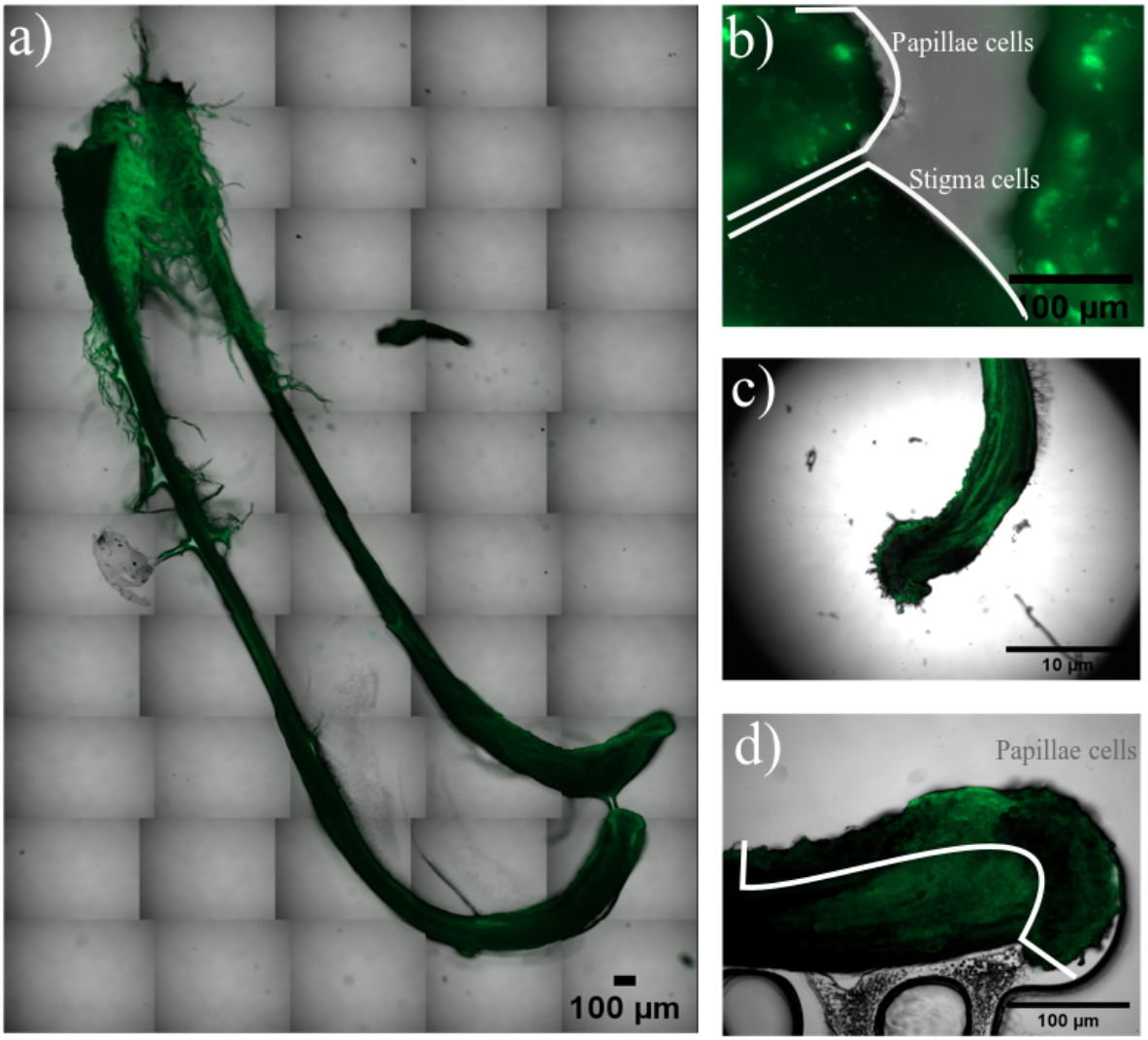

Figure 12. *P. agglomerans* on the stigma and style after incubation for 24 hours. These samples were fixed as described in section 4.2.1. a) shows a stigma and style overview. b) shows a stigma at the junction of papillae cells and stigma cells. d) is a profile view of the stigma.

Samples that underwent fixation have greater autofluorescence than unfixed sampled and have a dehydrated and shrivelled appearance as can be seen in Figure 12. Very few

populations of *P. agglomerans* were observed after 24 hours, and these populations were located on the papillae cells as shown in image b), Figure 12..

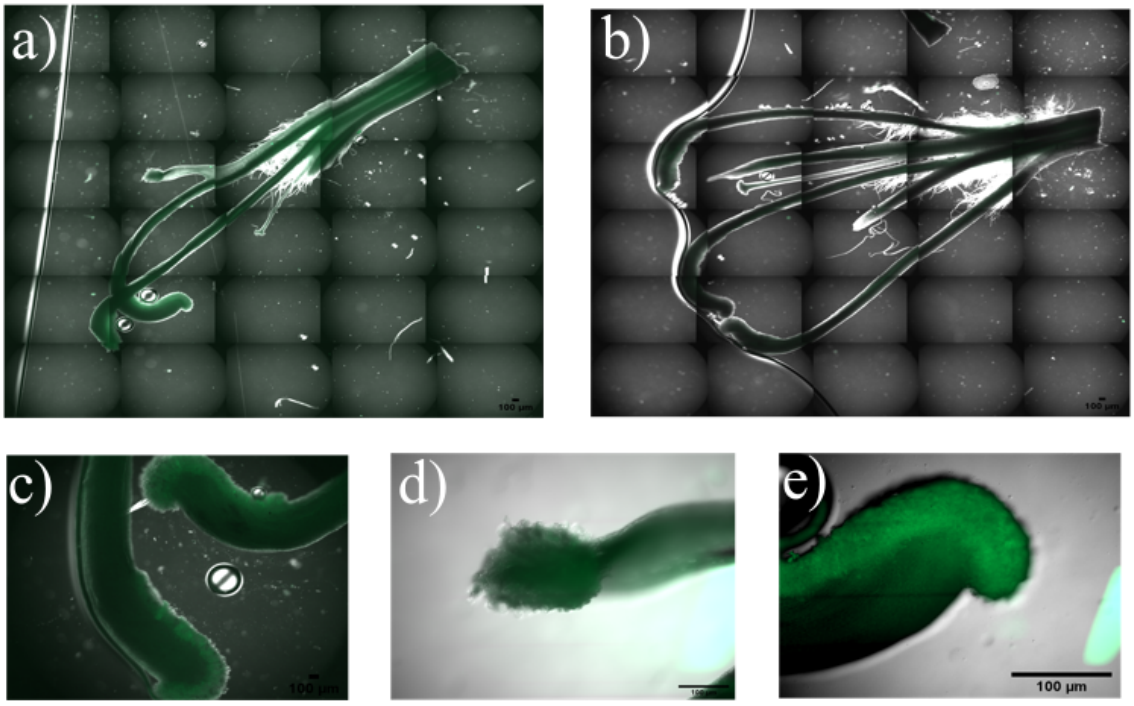

Figure 13. *P. agglomerans* on the apple blossom stigma and style incubated for 48 hours after inoculation as a monoculture. These blossom stigma samples were fixed prior to microscopy. a) and b) are overviews of the stigma and style. c), d), and e) show the stigma at higher magnifications.

There are no observable *P. agglomerans* populations after 48 hours of incubation (Figure 13).

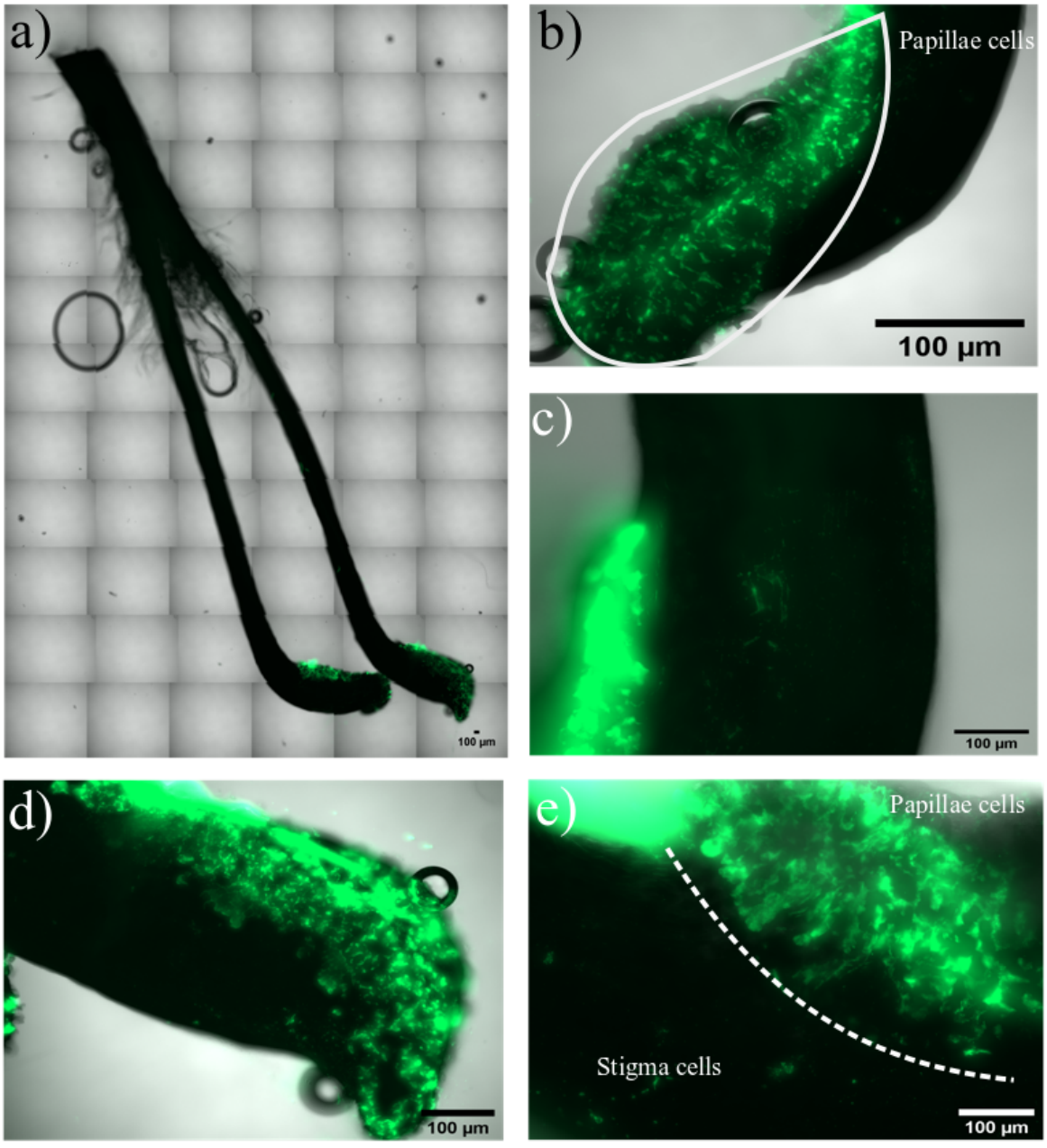

Figure 14. ***P. agglomerans*** **on the apple blossom stigma and style incubated for 72 hours after inoculation as a monoculture. a) shows an overview of the stigma and style. b) shows a close up of** ***P. agglomerans*** **on the papillae cells and the adjacent stigma cells. c) shows** ***P. agglomerans*** **on the stylar surface at the edge of the papillae cells. d) shows the**

stigma and papillae cells. e) shows the papillae and stigma cells at a greater magnification than image d).

After 72 hours of incubation *P. agglomerans* is growing densely on the papillae cells and has begun to grow in the stylar groove as seen in Figure 14. Populations of *P. agglomerans* are observable between the papillae cells and are very fluorescent suggesting dense populations (Figure 14).

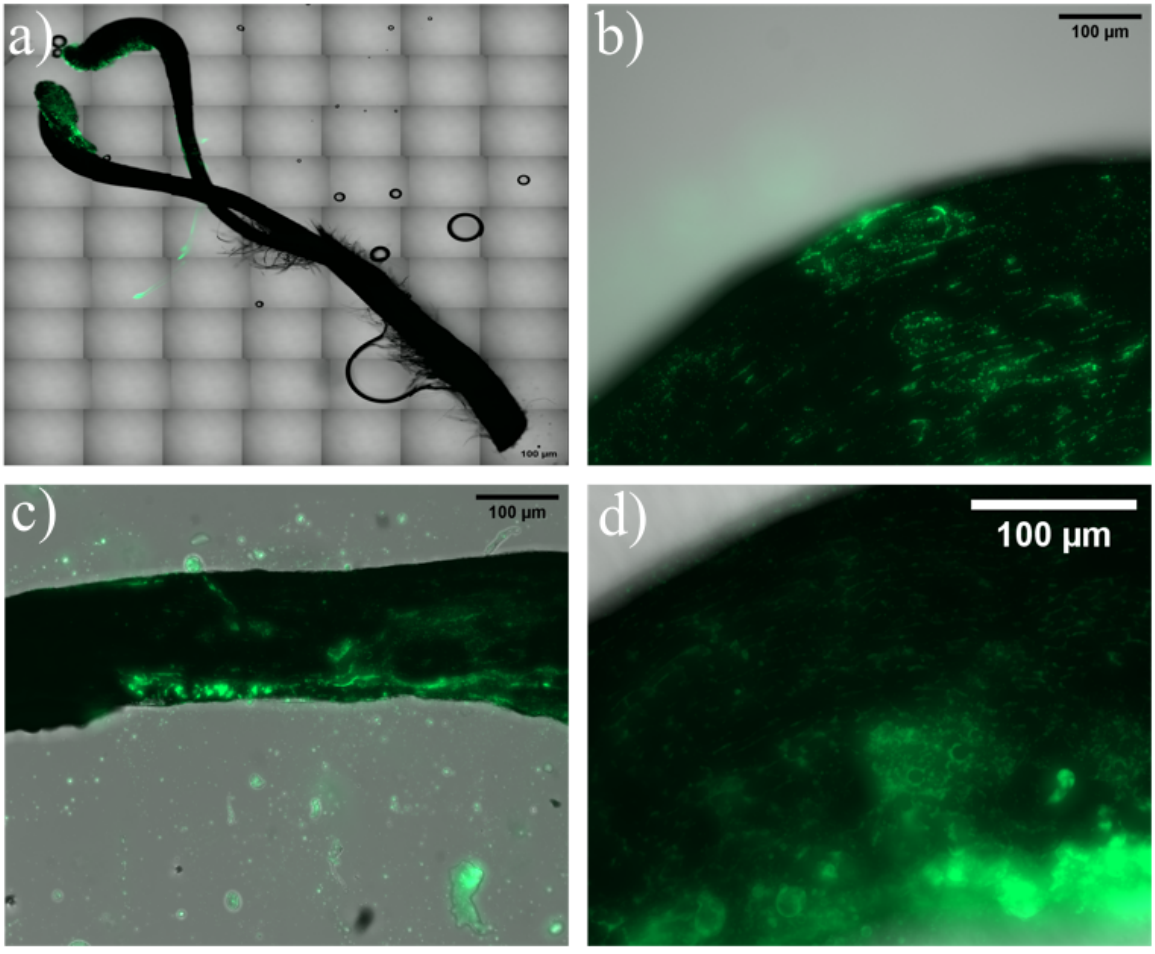

Figure 15. *P. agglomerans* on the apple blossom stigma and style incubated for 96 hours after inoculation as a monoculture. a) shows an overview of the stigma and style. b) shows *P. agglomerans* on the style cells. c) shows *P. agglomerans* in the stylar groove. d) shows *P. agglomerans* on the stigma cells and the papillae cells.

After 96 hours, *P. agglomerans* was primarily observed on the papillae cells of the stigma. *P. agglomerans* was observed in the stylar groove showing similar progression from the stigma towards the hypanthium as *E. amylovora* (image c), Figure 15). Small groups of *P. agglomerans* cells were observable on the stigma cells, although as can be observed in image d) these are less intensely fluorescent suggesting smaller populations. Populations of *P. vagans* on the stigma and style cells are clustered on the plant cell margins (images b) and d).

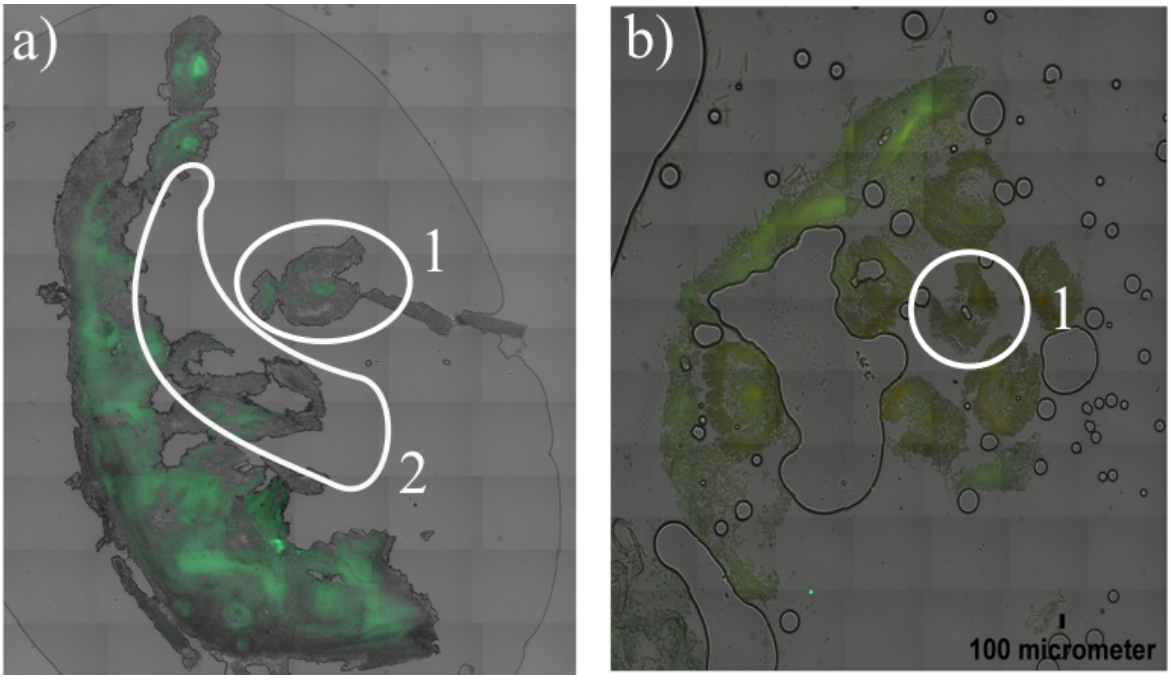

Figure 16. Hypanthium from blossoms inoculated with *P. agglomerans* monoculture. 1 indicates the blossom style, 2 indicates the nectarthode cells.

There is no evidence of *P. agglomerans* on the hypanthium cells after incubation for 96 hours (Figure 16).

4.3.3.2 *P. agglomerans* and *E. amylovora* inoculated onto stigma and style as a co culture

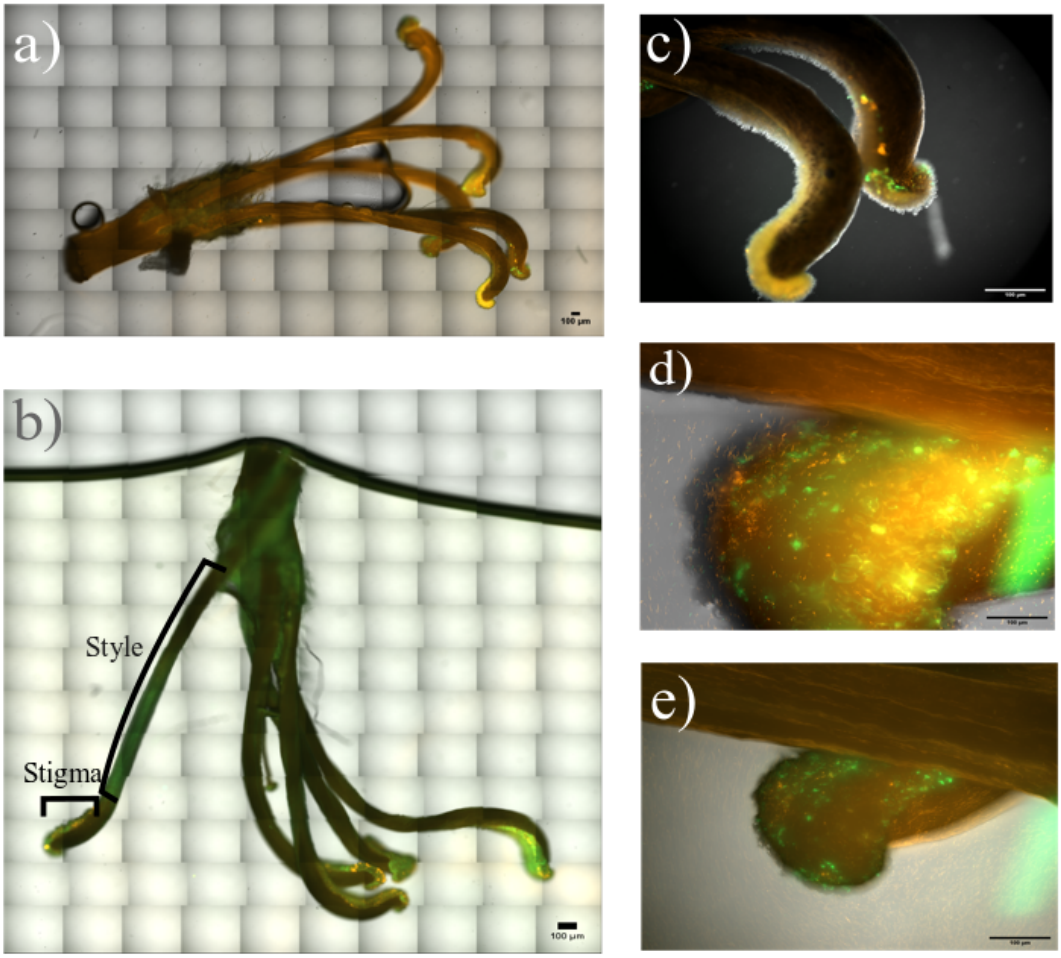

Figure 17. *E. amylovora* and *P. agglomerans* on apple blossom stigma and style after incubation for 24 hours. a) and b) are stigma and style overview images. c) shows a profile view of the stigma and part of the style. d) shows the tip of the papillae cells, shown from under the top of the stigma. e) shows the papillae cells and a small area of the stigma cells. There is a style overlaying this stigma.

Both *P. agglomerans* and *P. vagans* are present on the papillae cells in small numbers. Areas of fluorescence are observed between the papillae cells, but they are not extensive and there

are many areas which have not been colonised. Images a), b) and c) in Figure 17 show that some stigma have observable fluorescence, while other stigmas have none.

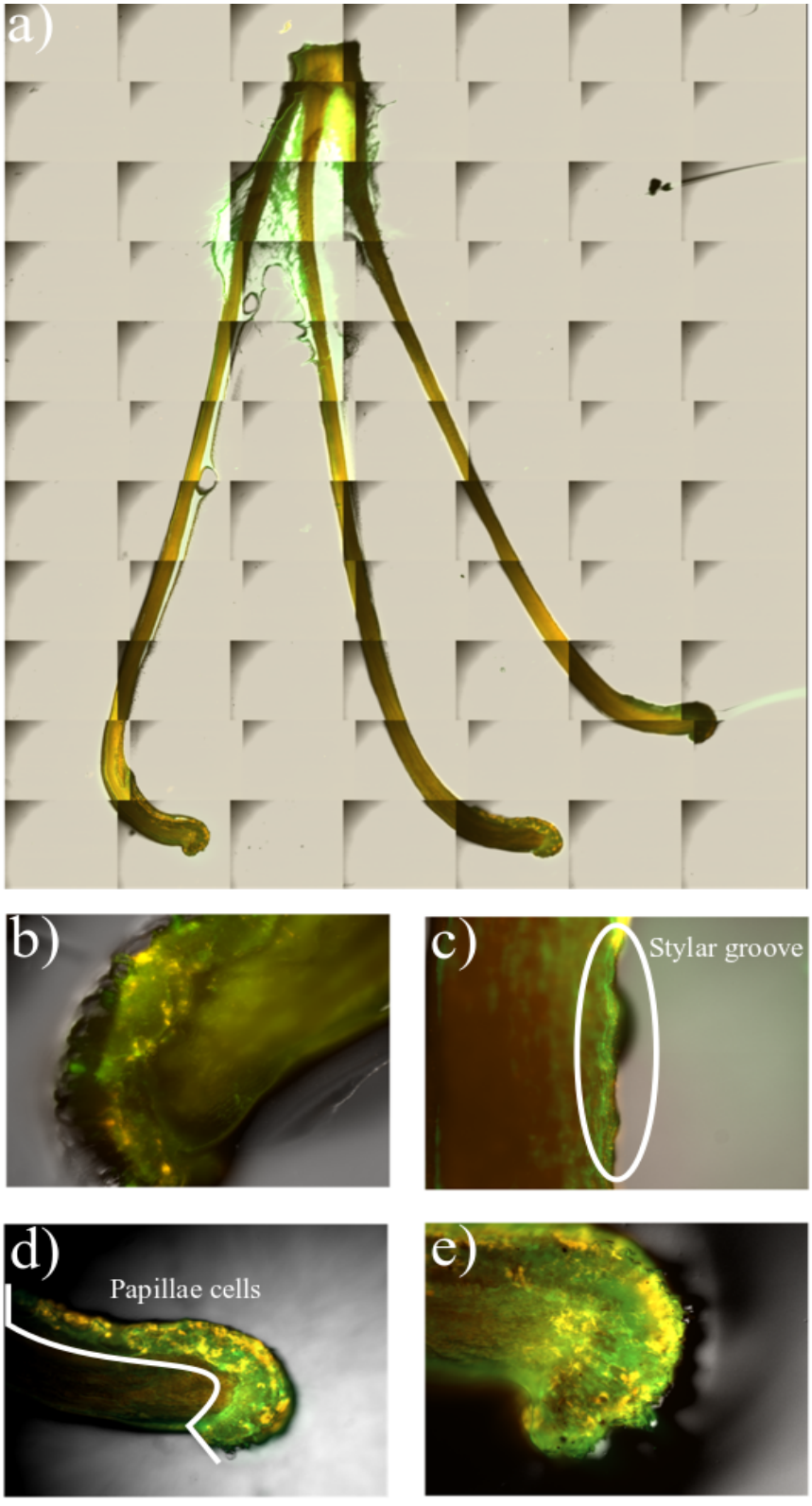

Figure 18. *P. agglomerans* and *E. amylovora* on the apple blossom stigma and style incubated for 48 hours after inoculation together. These samples were fixed prior to microscopy. a) is an overview of the stigma and style. b), d) and e) are profile views of the stigma. c) shows the style with the stylar groove on the right hand side.

P. agglomerans and *E. amylovora* both colonise the papillae cells of the stigma as seen in Figure 18. Both strains were also observed on the style of the blossom in the stylar groove, although this was unusual among the samples (image c), Figure 18). As these samples were fixed, and it is known the fixation process removed bacterial cells, I expect the fluorescence intensity has been reduced on these samples.

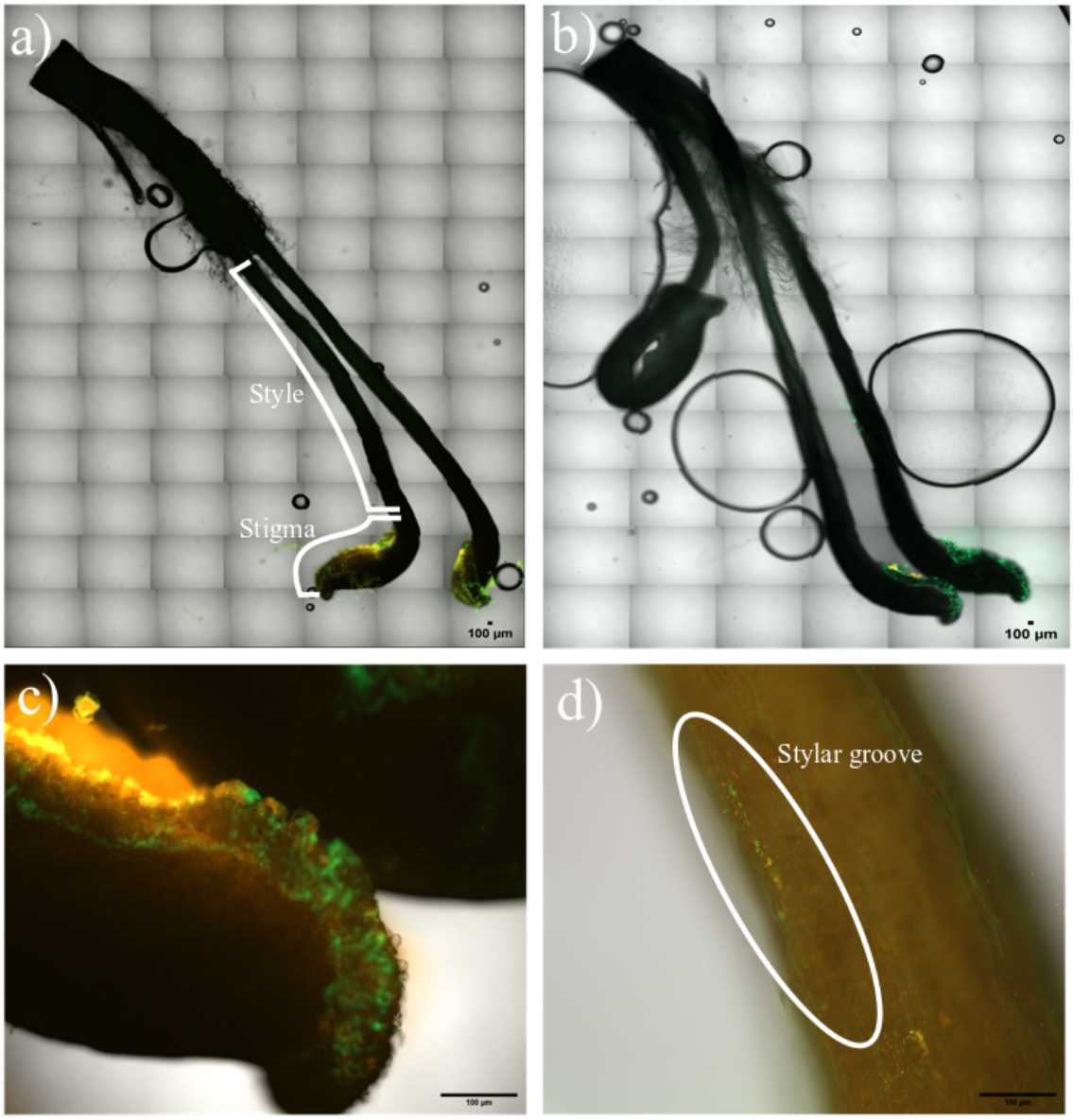

Figure 19. *P. agglomerans* and *E. amylovora* on the apple blossom stigma and style incubated for 72 hours after inoculation together. a) and b) are overviews of the stigma and style. c) shows the tip of the stigma. d) shows the stylar groove with fluorescent bacteria circled.

After 72 hours of incubation papillae cells have been heavily colonised by *P. agglomerans* and *E. amylovora* as shown in images a) and b). Some stigma are primarily colonised by *E.*

amylovora while others show a greater proportion of the population as *P. agglomerans* suggesting a niche overlap between the two strains rendering them incompatible on the papillae cells (Figure 19).

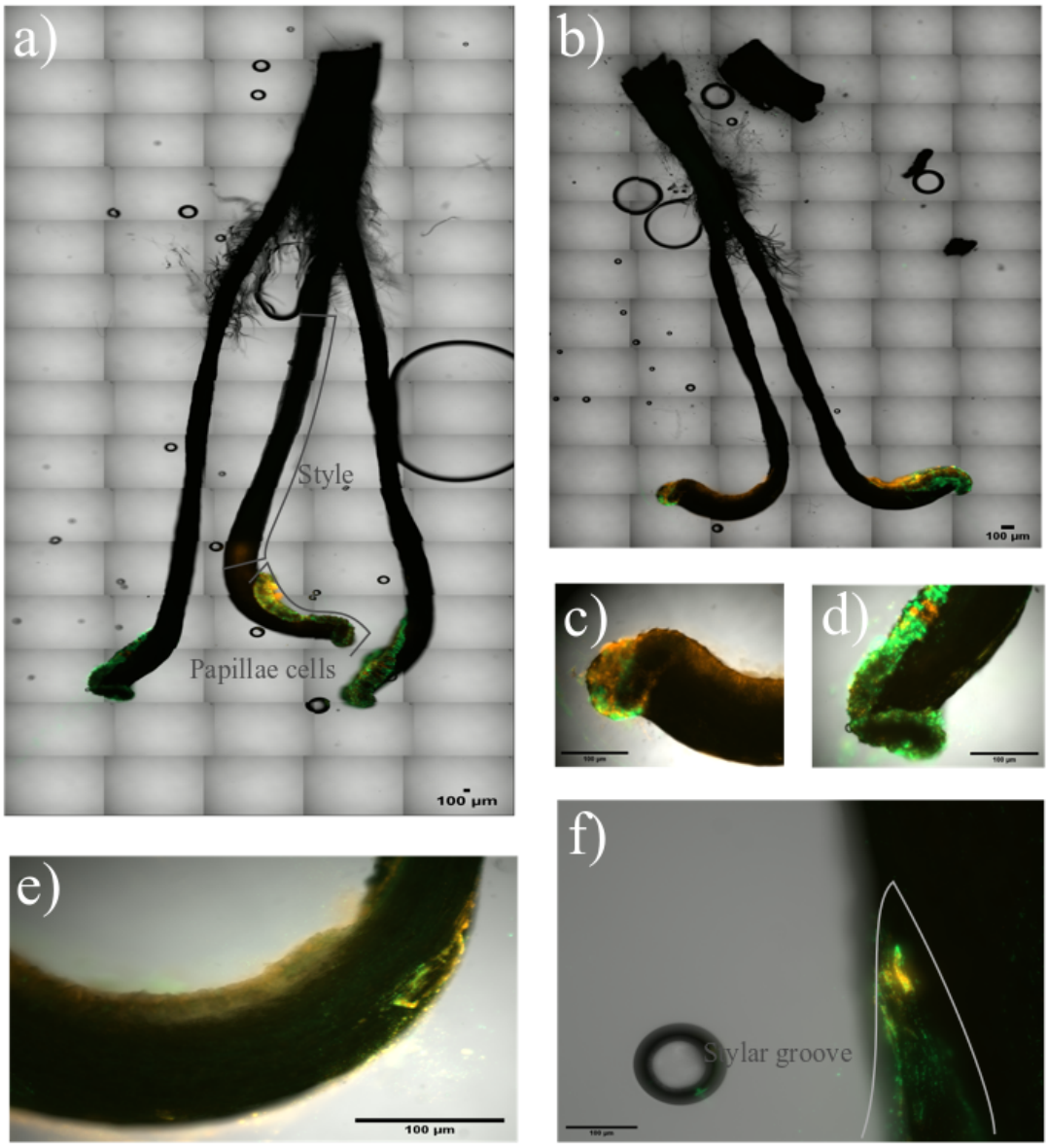

Figure 20. *P. agglomerans* and *E. amylovora* on the apple blossom stigma and style incubated for 96 hours after inoculation together. Images a) and b) show overviews of the stigma and style. c) and d)stigma and papillae cells colonised by *P. agglomerans* and *E. amylovora*. e) and f) show *P. agglomerans* and *E. amylovora* on the style

E. amylovora and *P. agglomerans* inoculated together both colonise the margins of the papillae cells, and migrate down the stylar groove towards the hypanthium. As can be shown in images c) and d) some stigma are primarily colonised by *P. agglomerans* or *E. amylovora* respectively (Figure 20). In this regard, the interaction between the strains is similar to that observed with *P. vagans* as the antagonist.

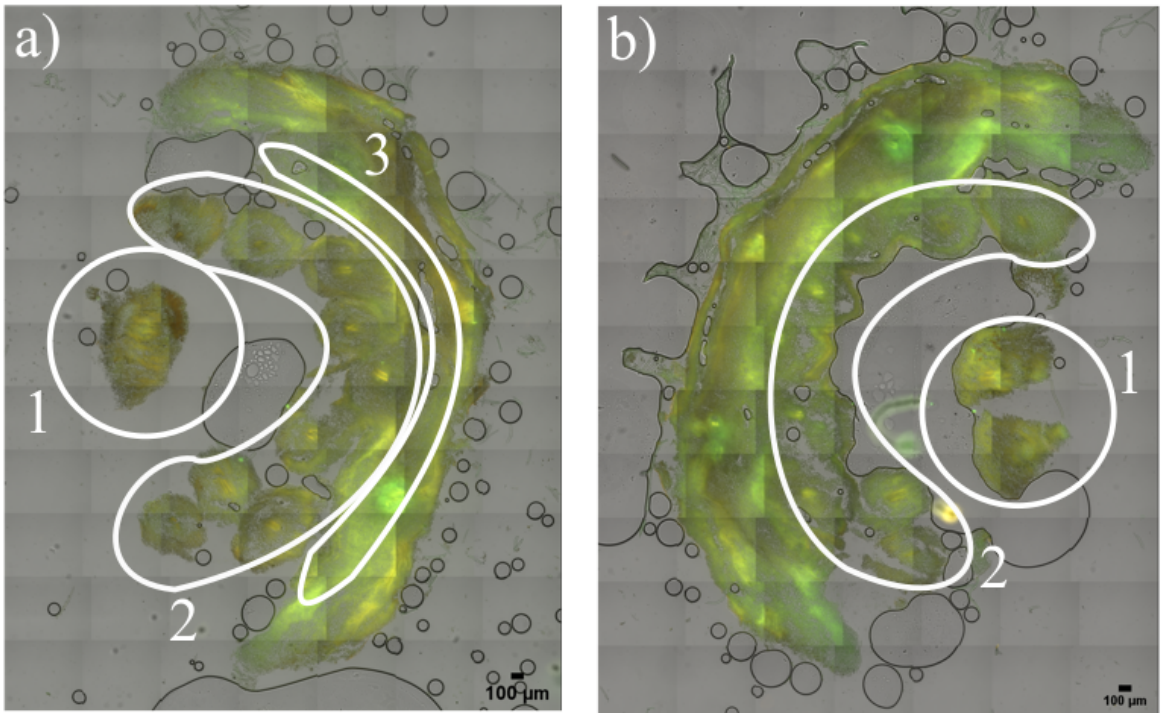

Figure 21. Hypanthium from blossoms inoculated with *E. amylovora* and *P. agglomerans* after incubation for 96 hours. 1 indicates the style, 2 indicates the anther filaments, 3 indicates the nectarthode cells.

No bacteria are observed on the hypanthium although there is some autofluorescence. The nectarthode cells do appear to be darkened compared to the rest of the plant tissue as is shown by image a) Figure 21.

In summary *P. agglomerans* and *E. amylovora* have a similar colonisation process on apple blossom stigma and style. *P. agglomerans* populations rapidly colonised the papillae cells of the stigma and after 72 hours of incubation was observed migrating towards the hypanthium. When *P. agglomerans* and *E. amylovora* were applied to the blossom together, there was no observed inhibition of *E. amylovora* growth or migration. As no bacteria were observed on the hypanthium after 96 hours of incubation, no conclusions can be made regarding interactions of *P. agglomerans* and *E. amylovora* on the nactarthode cells. *P. agglomerans* and *E. amylovora* reached larger population sizes with greater incubation time as shown by the detached flower assay (Chapter 3) therefore as no bacteria were present after this length of incubation, observations of samples incubated for less time were considered unnecessary.

4.3.4 Interactions of *S. melonis* and *E. amylovora*

This section presents microscopy results demonstrating changes to apple blossom colonisation by *E. amylovora* and *S. melonis* populations when the strains are inoculated together. Section 4.3.4.1 shows the results of apple blossom stigma and styles inoculated with *S. melonis* and incubated for 24, 48, 72, and 96 hours as well as the apple blossom hypanthium after incubation for 96 hours. Section 4.3.4.2 shows the results of apple blossom stigma and styles inoculated with *S. melonis* and *E. amylovora* and incubated for 24, 48, 72, and 96 hours as well as the apple blossom hypanthium after incubation for 96 hours. As part of image processing *S. melonis* fluorescence was labelled green despite emitting yellow fluorescence so that in all images, green fluorescence indicates the presence of the antagonist.

4.3.4.1 *S. melonis* inoculated as a monoculture

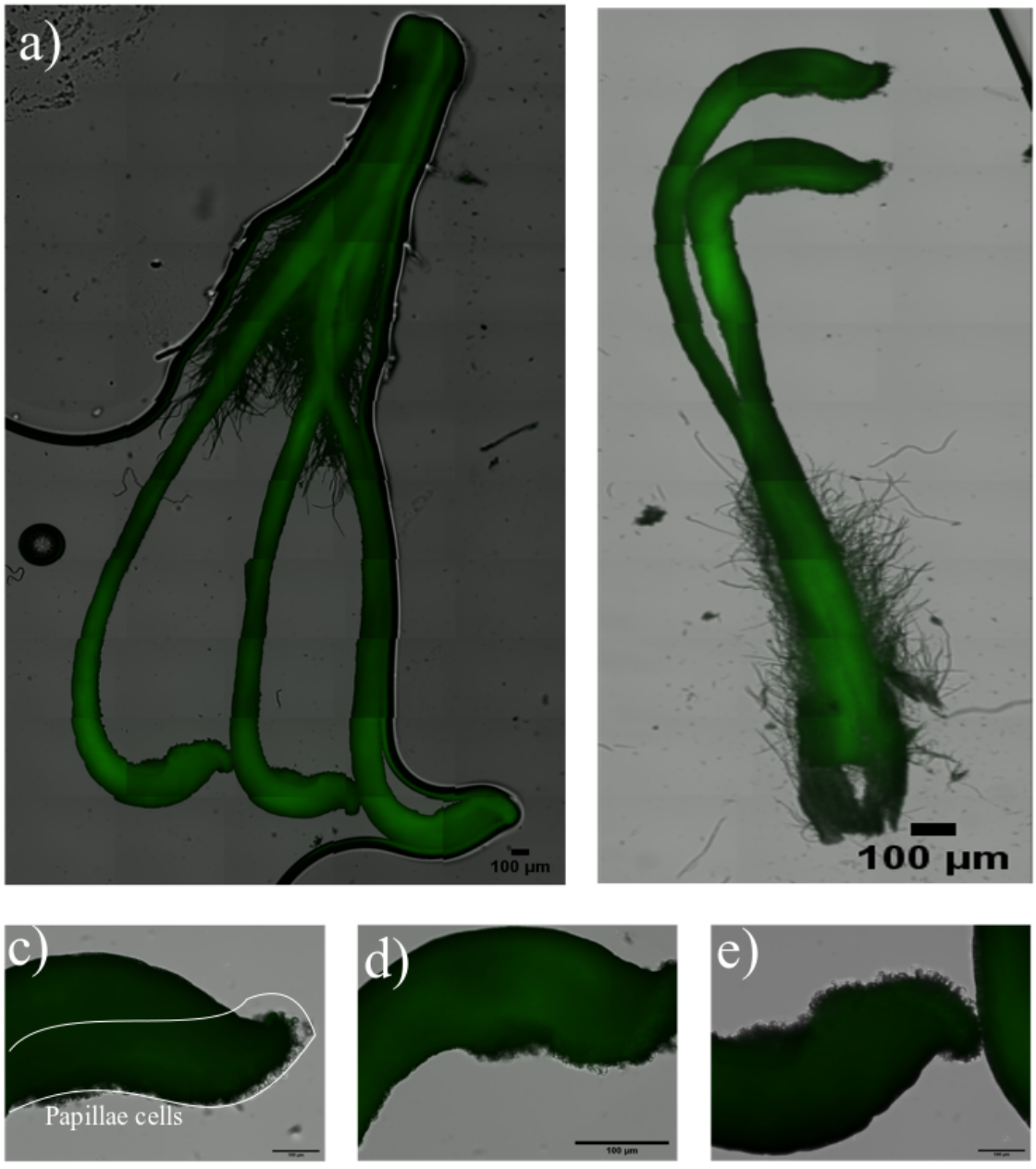

Figure 22. *S. melonis* on the stigma and style after incubation for 24 hours. These samples were fixed before microscopy. a) and b) are overview images of stigmas and styles. c) d) and e) are profile images of the stigma.

There are no observable cells of *S. melonis* present on the stigma and style after 24 hours of incubation as shown in Figure 22. As these samples underwent fixation before microscopy, it is not unexpected to see no bacterial cells as initial investigations found the fixation process removed bacterial cells.

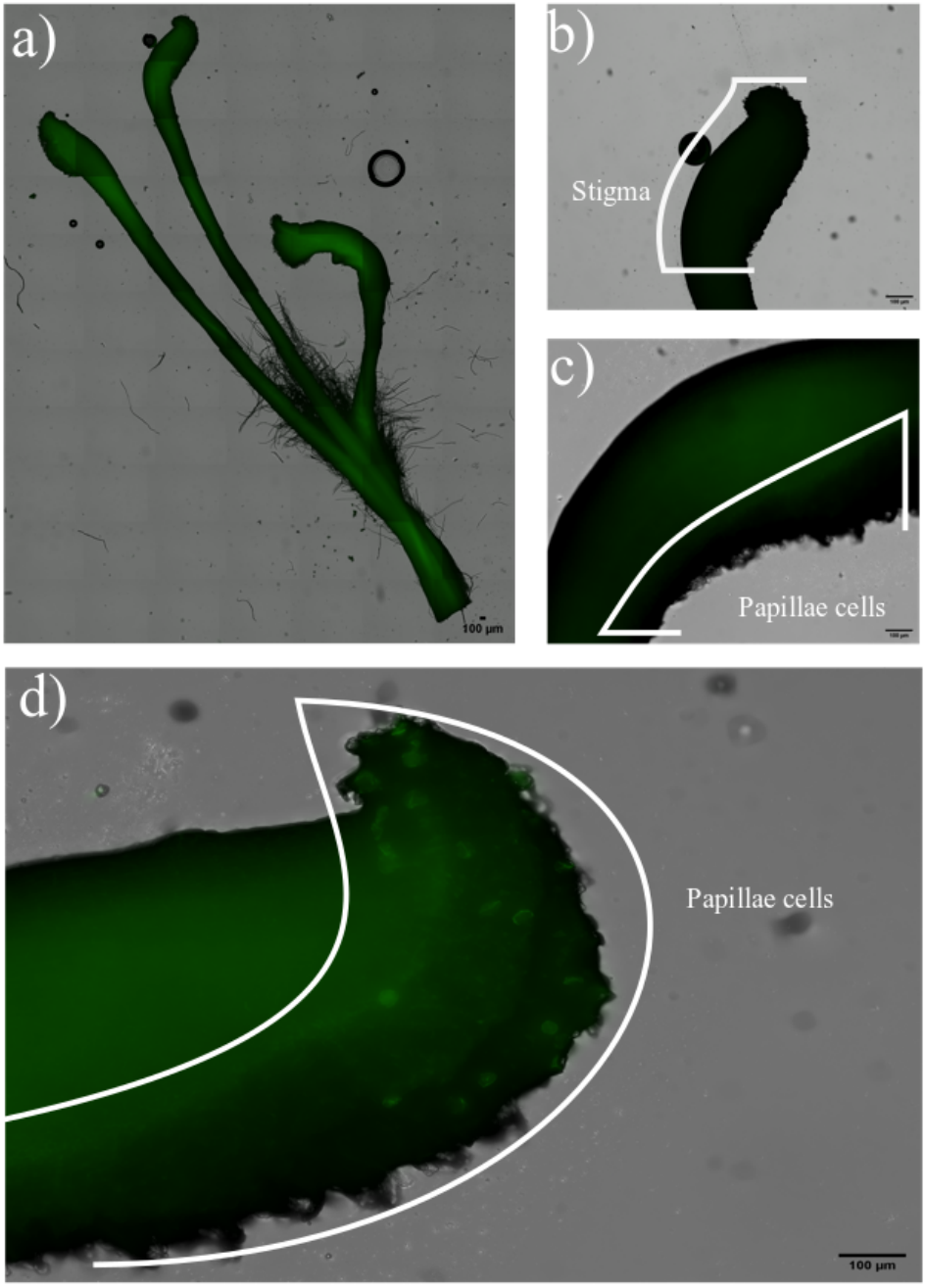

Figure 23. *S. melonis* on apple blossom stigma and style after incubation for 48 hours. These samples were fixed before microscopy. a) an overview of the stigma and style. b) the stigma. c) the lower half of the stigma at the junction with the style. d) the tip of the stigma.

There are no *S. melonis* observed on the stigma and style after incubation for 48 hours as shown in figure 23.

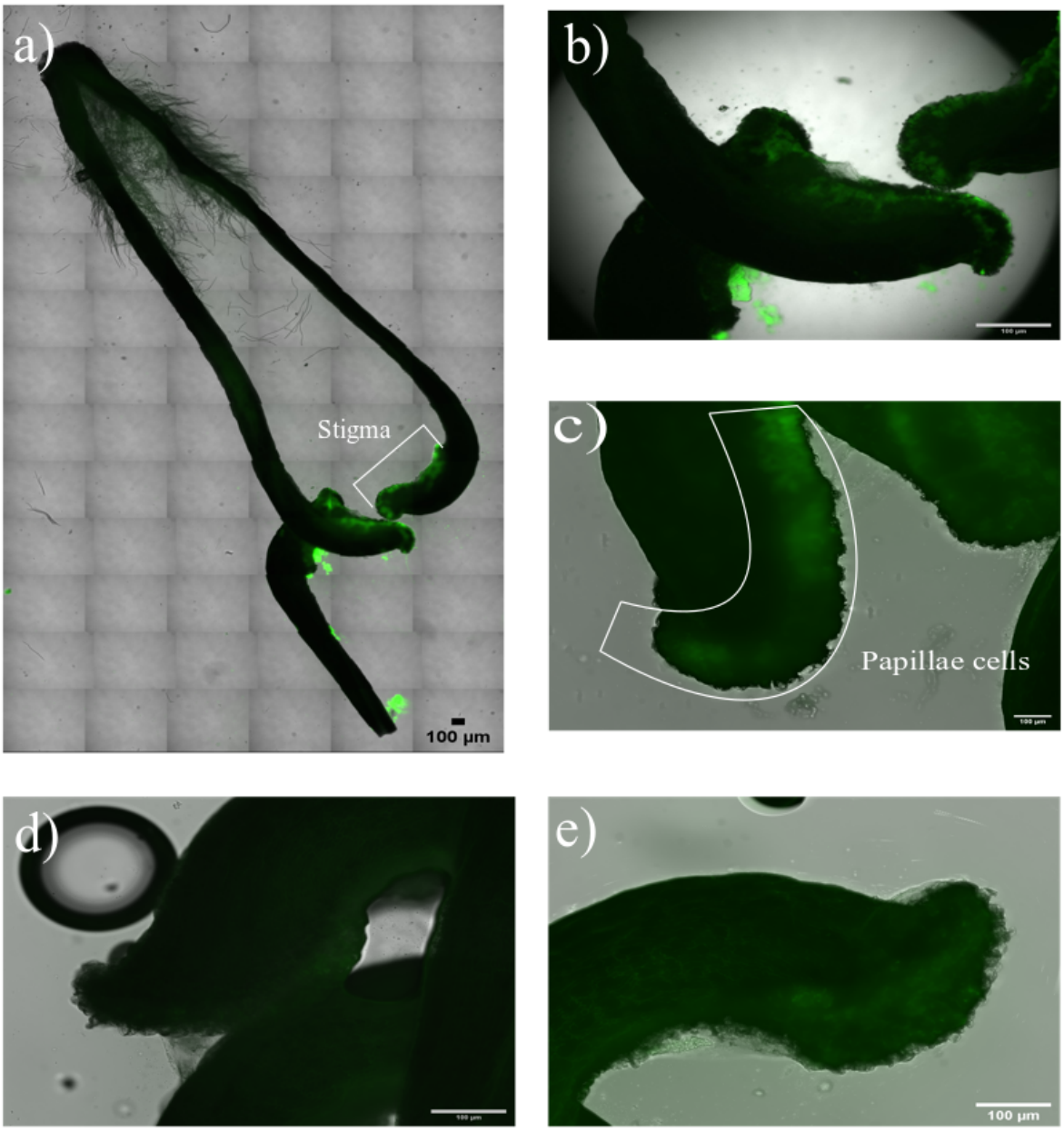

Figure 24. *S. melonis* on apple blossom stigma and style after incubation for 72 hours. a) is an overview of the stigma and style. b) multiple stigma. c) profile perspective of the stigma with papillae cells indicated. d) and e) profile perspective of the stigma.

After 72 hours there are observable populations of *S. melonis* as shown in image a), Figure 24. *S. melonis* primarily resides on the papillae cells of the stigma. Image a) shows bacteria

on the style, but as these were not attached to the style they are likely transferred from the stigma during microscopy slide preparation. *S. melonis* were less fluorescent than the *P. vagans*, *P. agglomerans* and *E. amylovora* strains, increasing difficulty detecting bacterial cells on autofluorescent plant samples. *S. melonis* cells dispersed into the ddH_20 water used to prepare slides as shown in image a), Figure 24.

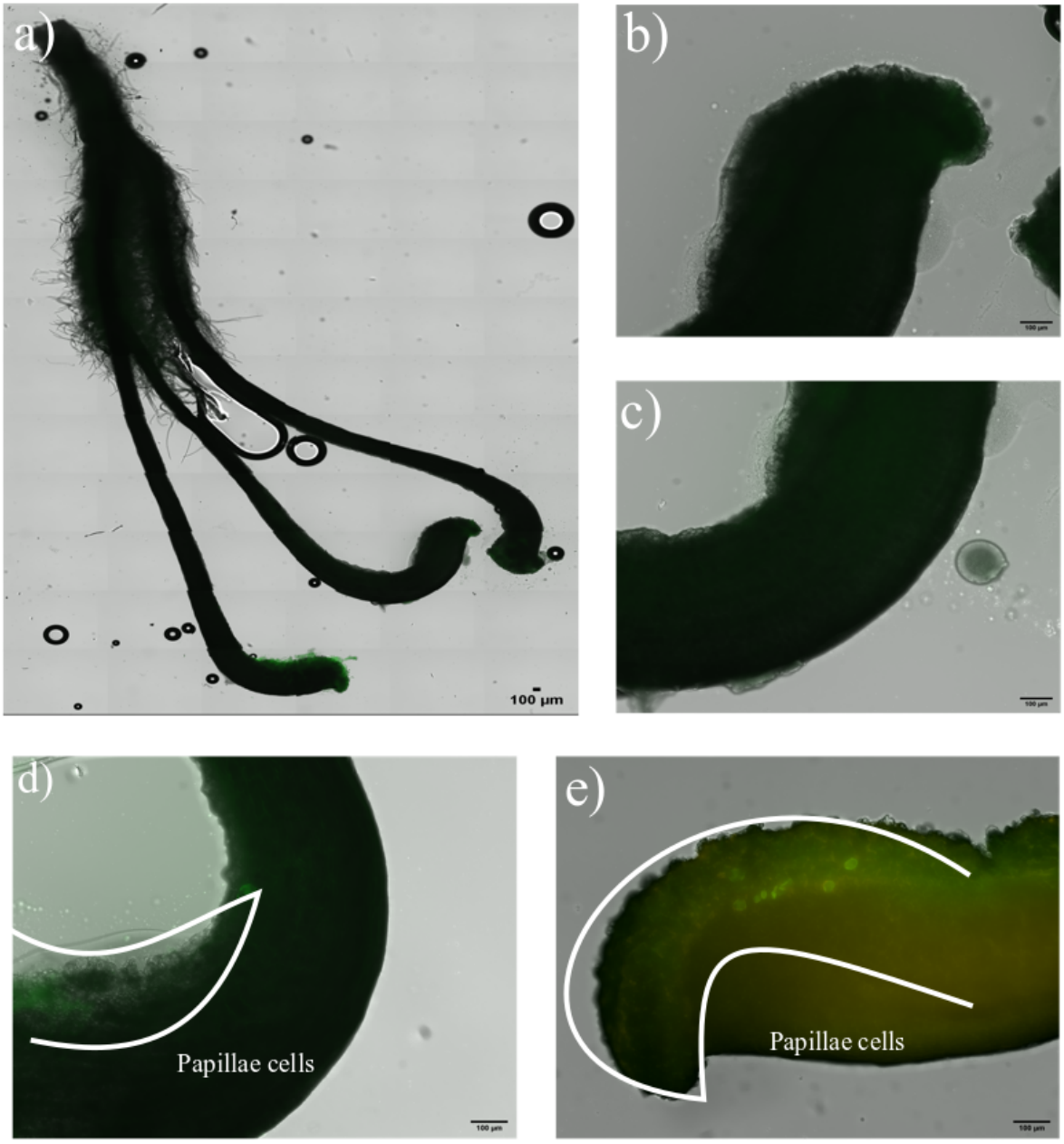

Figure 25. *S. melonis* on apple blossom stigma and style after 96 hours of incubation. a) shows an overview of the stigma and style. Images b) and e) show a profile view of the stigma tip including the papillae cells. c) and d) show a profile view of the lower half of the stigma at the junction with the style.

Fluorescent bacteria observed on the 96 hour samples were not observed on all stigma and had a lower fluorescence intensity than *P. vagans* and *P. agglomerans.* Populations of *S. melonis* are primarily observed on the papillae as shown in images a) d) and e) (Figure 25). Compared to *S. melonis* populations observed after incubation for 72 hours, there is reduced fluorescence intensity is reduced suggesting smaller population sizes.

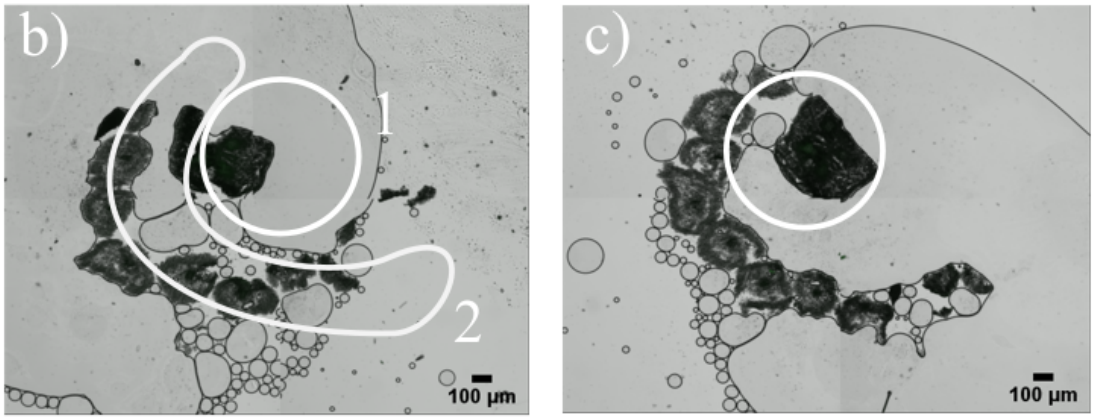

Figure 26. Hypanthium from blossoms inoculated with an *S. melonis* monoculture and incubated for 96 hours. 1 indicated the style, 2 indicates the anther filaments.

There are no *S. melonis* cells observed on the hypanthium after 96 hours as shown by Figure 26.

4.3.4.2 *S. melonis* and *E. amylovora* inoculated on stigma and styles

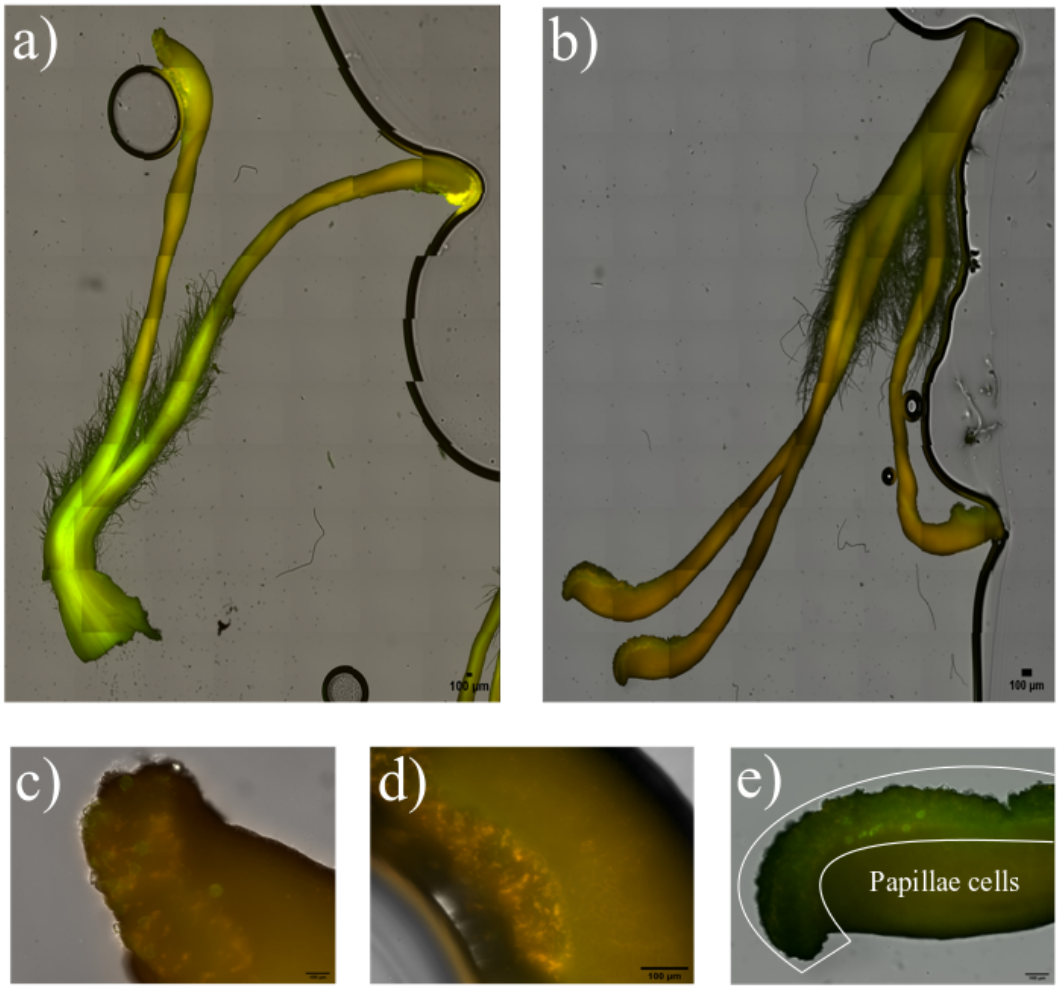

Figure 27. *E. amylovora* and *S. melonis* on stigma and style after incubation for 24 hours. a) and b) show overviews of stigmas and styles from single blossoms. c) Papillae cells from a profile perspective. d) Papillae cells closer to the hypathium compared to image c). e) a stigma from a profile perspective.

There is a lot of autofluorescence in these samples which hinders visualising the bacterial populations on the papillae cells. There is low fluorescence of these populations, indicating a small population size. The green fluorescence seen in figure e) is highly fluorescent single

papillae cells not *S. melonis*. *E. amylovora* after 24 hours of incubation is located between the papillae cells (images c) and d),Figure 27).

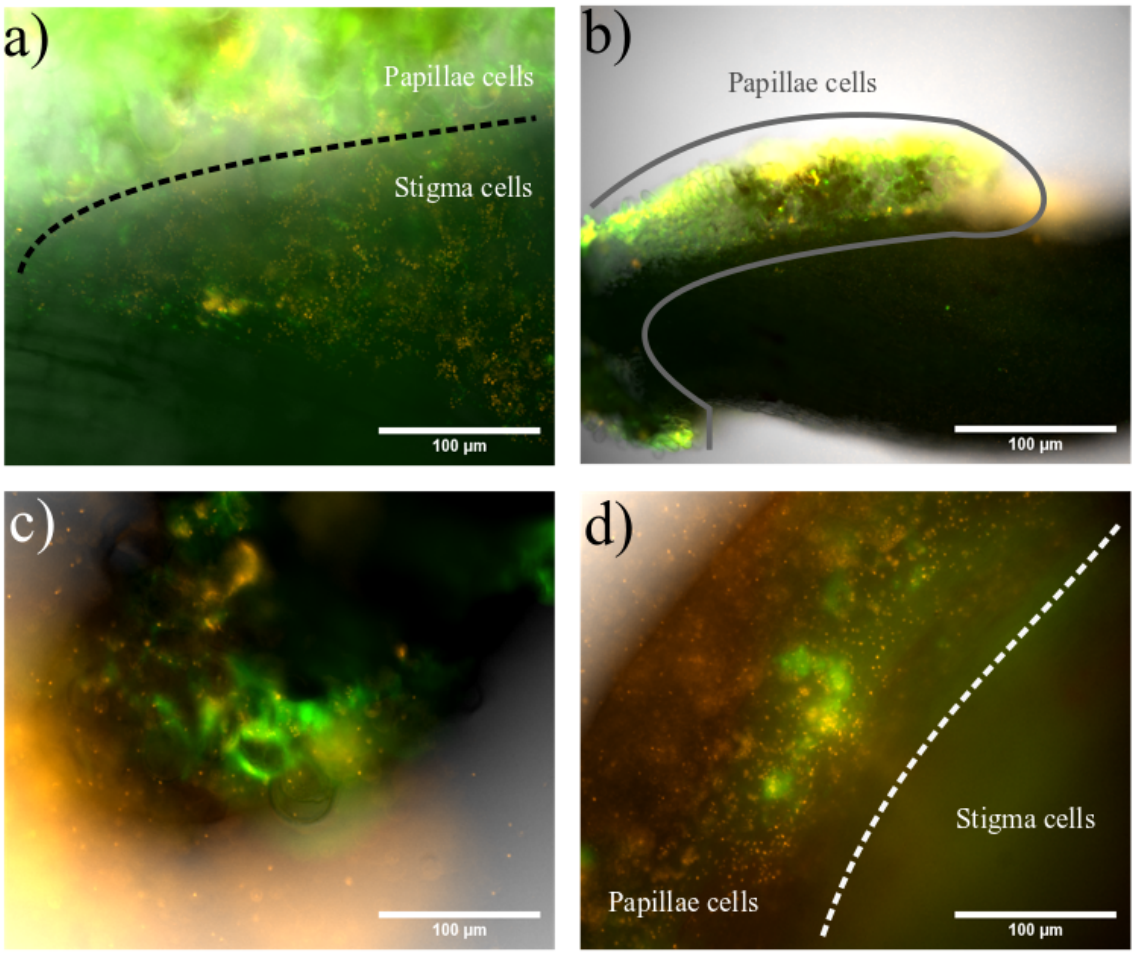

Figure 28. *E. amylovora* and *S. melonis* on apple blossom stigma and style after incubation for 48 hours. a) and d) show the papillae and stigma cells. b) shows the stigma. c) shows the stigma from the outer edge of the style.

After 48 hours of incubation *E. amylovora* began to diffuse into the ddH_20 water used to prepare slides as shown in images c) and d) (Figure 28). *S. melonis* populations appear

brightly fluorescent and clustered in small groups as shown in images c) and d) (Figure 28). Image a) shows both *E. amylovora* and *S. melonis* present on the stigma cells (Figure 28).

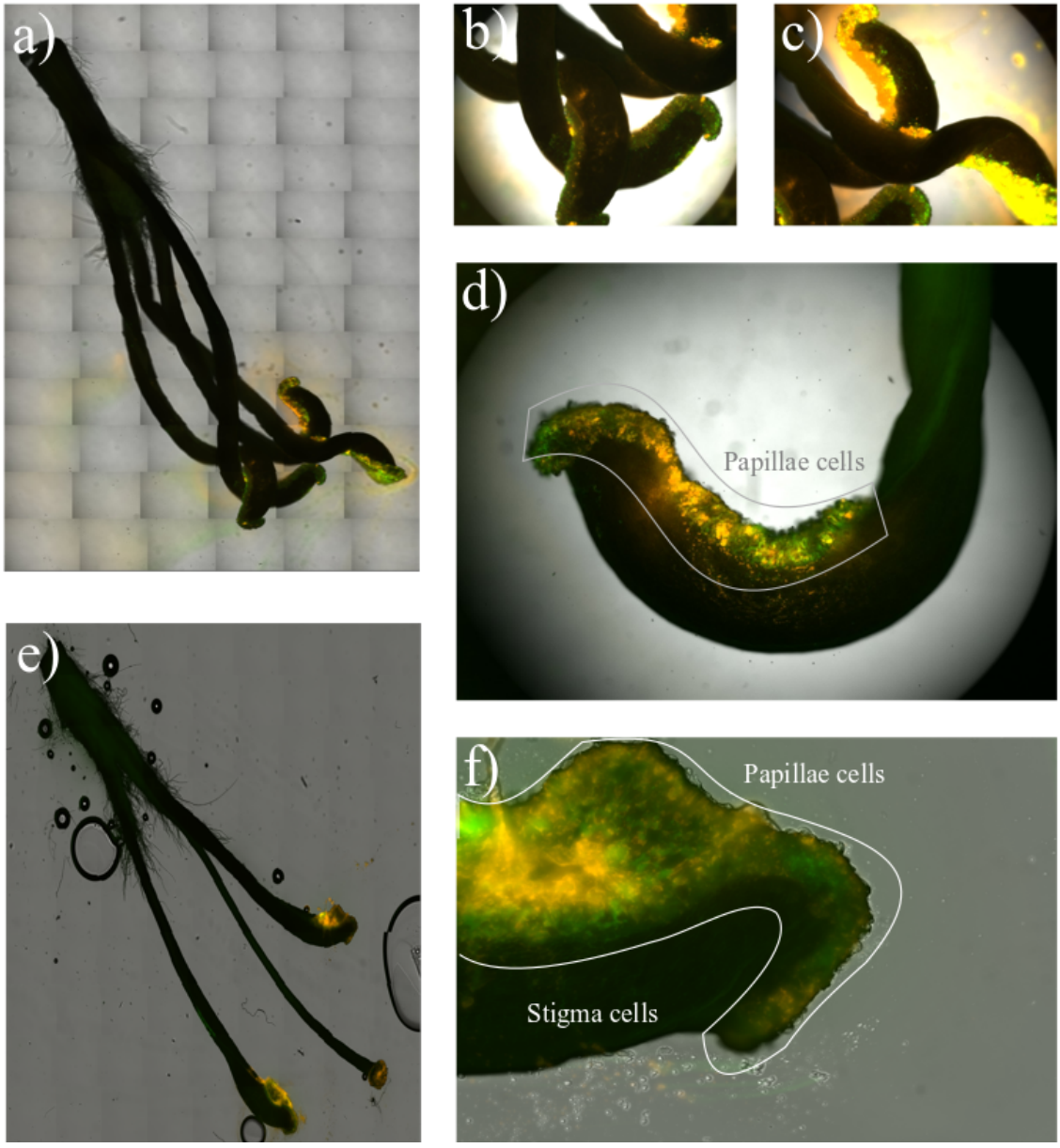

Figure 29. *E. amylovora* and *S. melonis* on apple blossom stigma after incubation for 72 hours. a) and e) are style and stigma over view images. b) and c) show multiple stigma entangled together. d) shows a profile perspective of a stigma with papillae cells. f) shows a stigma viewed from the underside of the stigma.

There are extensive populations of *E. amylovora* observed on the papillae cells, but no evidence these populations have begun to move down the style towards the hypanthium. Populations of *S. melonis* are observed on the papillae cells. Figure d) shows individual cells of *E. amylovora* and *S. melonis* on the junctions of stigma cells.

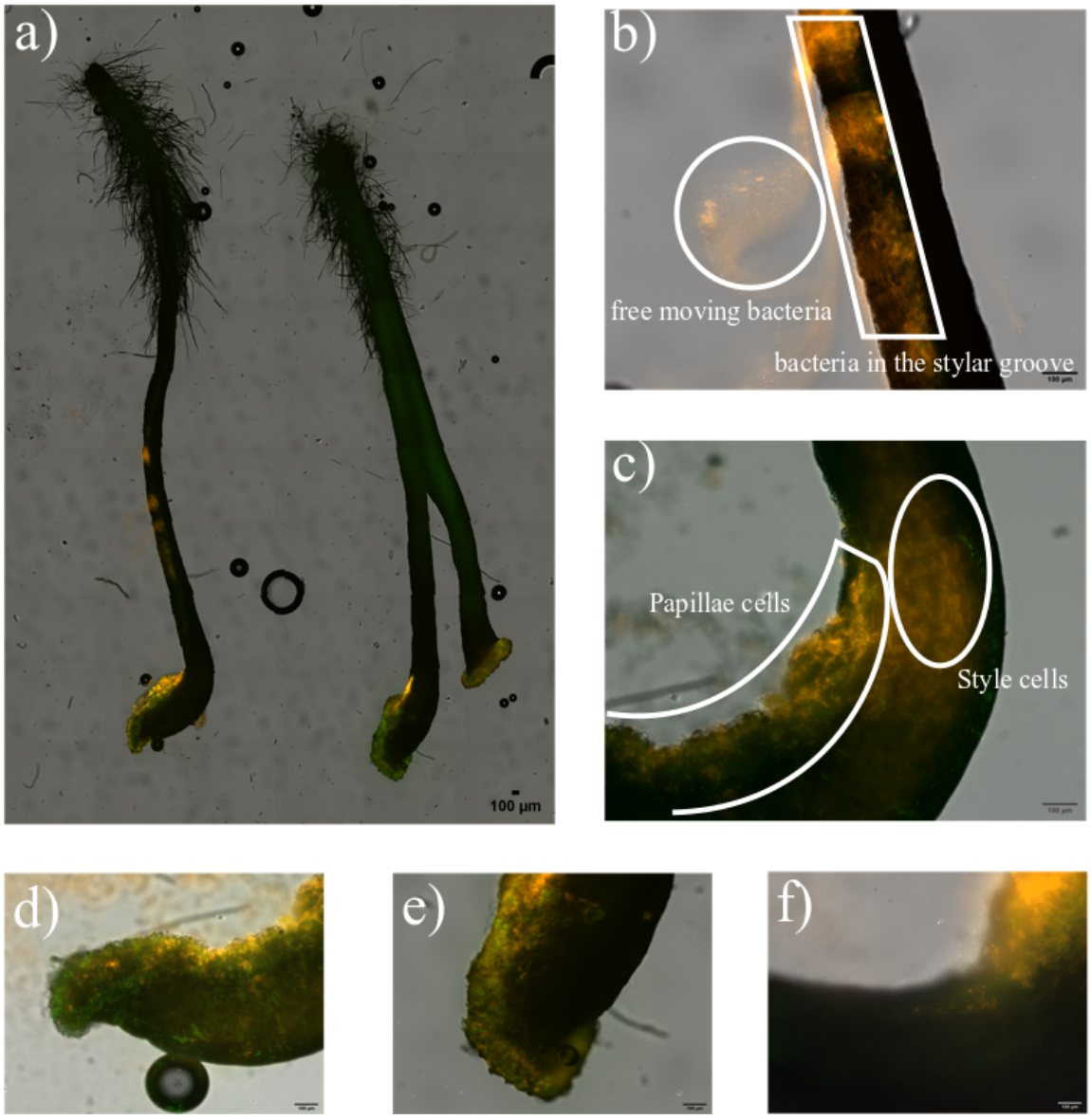

Figure 30. *E. amylovora* and *S. melonis* on stigma and style after incubation for 96 hours. a) shows an overview of the stigma and style. b) shows *E. amylovora* in the stylar groove. c) is a profile view image of the base of the papillae cells at the junction with

style cells. d) The tip of the style with papillae and stigma cells from a profile perspective. e) the stigma from a birds- eye perspective. f) the base of the papillae cells at the junction with the style cells.

The papillae cells of the stigma are heavily colonised by both *E. amylovora* and *S. melonis*. As can be seen in images a), c), and f), *E. amylovora* on the papillae cells is closer to the style cells than *S. melonis* (Figure 30). *E. amylovora* was observed in the stylar groove as can be seen in images a) and b) (Figure 30).

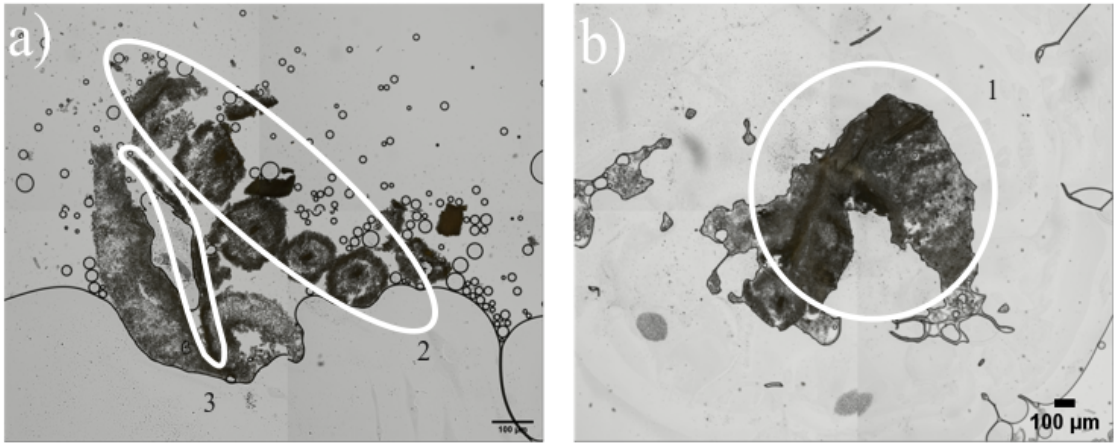

Figure 31. Hypanthium of blossoms colonised by *E. amylovora* and *S. melonis*. 1) indicates the style, 2 indicates anther filaments, 3 indicates nectarthode cells.

Image a) shows the nectarthode cells are darker than the rest of the hypanthium tissue suggesting these cells have degraded more than the rest of the hypanthium (Figure 31). There were no *S. melonis* or *E. amylovora* cells observed although in image b) there is an autofluorescent particle next to the style (Figure 31).

In summary *S. melonis* fluorescence was less intense that the fluorescence of *P. vagans*, *P. agglomerans*, and *E. amylovora* strains which made detection of *S. melonis* against an

autofluorescent plant surface more difficult. After 72 hours of incubation *S. melonis* has colonised stigma primarily on the papillae cells, although not all stigma were colonised successfully. After 96 hours of incubation a decrease in the fluorescence intensity indicates the populations of *S. melonis* have decreased. *S. melonis* when applied with *E. amylovora* was observed on the papillae cells as small dense aggregates after 48 hours of incubation (Figure 28). With increasing incubation time, the papillae cells were fully colonised by *S. melonis*, despite existing *E. amylovora* populations. As no bacteria were observed on the hypanthium after 96 hours of incubation, no conclusions can be made regarding interactions of *S. melonis* and *E. amylovora* on the nectarthode cells. *S. melonis* and *E. amylovora* reached larger population sizes with greater incubation time as shown by the detached flower assay (Chapter 3) therefore as no bacteria were present after this length of incubation, observations of samples incubated for less time were considered unnecessary.

4.3.5 Summary

The papillae was found to host the greatest population sizes, with high fluorescence intensity compared to the stigma cells, style, and hypanthium. Bacterial cells were also commonly observed on the style and stigma cells, although at very low densities compared to populations on the papillae. Single cells could be observed on the junctions of stigma and style cells unlike the papillae cells on which populations were too dense for the fluorescence of single bacterial cells to be detected. I do not consider the fluorescence of the papillae cells to be the result of plant tissue autofluorescence, because in many samples, the papillae were found to have reduced or no observable fluorescence, therefore I conclude that observed fluorescence is due to the presence of bacteria (Figure 3 a)). Samples which had undergone

fixation did show much greater autofluorescence than samples which were not fixed and these samples has bacteria washed away by the fixation process.

4.4 Discussion of Microscopy results

4.4.1 *E. amylovora* colonisation as a mono culture

Growth of *E. amylovora* was primarily on the papillae cells of the stigma. The intercellular spaces of the stigma is known to support high populations of microbes due to secreted sugars, amino acids, and high humidity therefore it is unsurprising to find dense populations in this area [1,25,35–38,42]. Additionally as collapse of the papillae cells occurs as blossoms age rendering the surface unable to be colonised by *E. amylovora*, growth between the papillae cells is considered an important step in development of *E. amylovora* population sizes sufficient for development of fire blight disease [25,47,48]. We see evidence in these results that large population sizes were only achieved between the papillae cells, with the stigma and style cells hosting much smaller populations, evident by the lower fluorescence intensity. These results provide evidence that if *E. amylovora* is unable to grow on the papillae the bacterial large population sizes required for development of fire blight are unlikely to occur. *E. amylovora* was observed in the stylar groove, supporting the work of Spinelli et al. 2005 which also found that the pathogen progressed towards the hypanthium within this area [51]. Populations in the stylar groove were small compared to those on the papillae cells predominantly in the plant cell margins.

4.4.2 *P. vagans* and *E. amylovora*

P. vagans was expected to migrate towards the stylar groove towards the hypanthium in the same way as *E. amylovora* based on previous studies [51]. However although *P. vagans* and *E. amylovora* both heavily colonise the papillae cells, *P. vagans* was only observed on some stigma. Additionally *P. vagans* did not grow to cover the papillae cells to the extent that *E.*

amylovora did. *P. vagans* was not observed in the stylar groove suggesting a different growth progression than *E amylovora*. These results do not support my hypothesis that: strains with an antagonistic effect against *E. amylovora* will colonise apple blossoms in the same way as the pathogen.

P. vagans colonisation of apple blossoms was expected to mirror *E. amylovora* activity based on previous studies identifying site exclusion as an important aspect of biocontrol, and studies which observed *P. vagans* in the stylar groove [51,96]. The results of this research found *P. vagans* exhibited different a different colonisation pattern to *E. amylovora*. *P. vagans* and *E. amylovora* after 96 hours of incubation were found to be spatially segregated suggesting these strains are incompatible, which is unsurprising given *P. vagans* is an effective antagonist of *E. amylovora* [29,85,97,98]. Segregation of *P. vagans* and *E. amylovora* allowed both strains flourish unchallenged, offering an explanation as to why co inoculation of antagonists with *E. amylovora* does not facilitate effective biocontrol [96]. As the nectarthode cells of the hypanthium are the site of *E. amylovora* invasion into the apple tree, antagonists that protect these sites against *E. amylovora* are of interest for biocontrol of fire blight [46]. The hypanthium had no observable *P. vagans* or *E. amylovora* cells although there was some darkening of the nectarthode cells, however it was not determined in these assays whether cell discolouration results from blossom ageing or the presence of inoculated strains.

4.4.3 *P. agglomerans* and *E. amylovora*

P. agglomerans colonisation of the stigma and style is primarily on the stigma between the papillae cells. *P. agglomerans* extensively colonised the papillae cells, and was observed in the stylar groove suggesting a colonisation process similar to *E. amylovora*. Effective biocontrol of *E. amylovora* is correlated with niche overlap and similarity in papillae

colonisation, therefore the results of this research suggest *P. agglomerans* will be an effective biocontrol strain.

Observing the activity of *P. agglomerans* and *E. amylovora* after application as a mixed inoculum did not show any evidence of *E. amylovora* inhibition by *P. agglomerans*. As was also observed in *E. amylovora* activity with *P. vagans*, *P. agglomerans* and *E. amylovora* spatially segregated on the stigma further supporting conclusions that these strains have similar resource requirements [96]. Further investigation of *P. agglomerans* 299R and its potential as an antagonist would assist in determining if *P. agglomerans* could be used for fire blight biocontrol. Blossoms inoculated with *P. agglomerans*, or a co- inoculation of both *P. agglomerans* and *E. amylovora*, had no bacteria observed on the hypanthium therefore no conclusions regarding the interaction of these strains on these surfaces can be made.

4.4.4 *S. melonis* and *E. amylovora*

Schlecter et al. 2018 found that plasmid borne fluorescence genes result in brighter cell fluorescence than chromosome borne genes for fluorescence [174]. This is supported by my observations that *S. melonis* fluorescence was less intense that *P. vagans, P. agglomerans* and *E. amylovora* fluorescence intensity. *S. melonis* lack of fluorescence increased the difficulty in detecting *S. melonis* on autofluorescent plant tissue. *S. melonis* after 48 hours was observed as small dense aggregates, but with additional incubation time grew to colonise areas of the stigma with disrupting existing *E. amylovora* populations. These observations suggest that *S. melonis* and *E. amylovora* colonises different areas of the papillae cells. Niche similarity is a predictor of antagonism therefore based on microscopy observations, *S. melonis* is unlikely to be an effective fire blight biocontrol method [96]. The hypanthium had no observable populations of *S. melonis* or *E. amylovora* although there was some darkening of the

nectarthode cells when the strains were applied simultaneously. As no bacteria could be observed, these assays could not determine whether cell discolouration results from blossom ageing or the presence of inoculated strains.

4.4.5 Summary

The papillae cells hosted the most dense populations of all strains as was shown by the fluorescence intensity compared to other areas with low cell populations such as the stigma cells. This is unsurprising given the papillae cells are known to secrete nutrients such as amino acids and sugars used by microbes for growth [1,35–38]. *P. vagans* is a known antagonist of *E. amylovora* and was considered a positive control to compare *P. agglomerans* and *S. melonis* activity to [29,85,97,98]. *S. melonis* colonisation was significantly different to colonisation by *P. vagans* and *P. agglomerans* suggesting antagonism of this strain will be mediated differently or reduced compared to *P. vagans*. Future analyses using alternative methods to observe *E. amylovora* and antagonists on the hypanthium would provide additional information regarding strain activity on this area of the blossom. Nectarthode cells in the hypanthium are the entry point for *E. amylovora* into the apple tree, therefore understanding any protective effect imposed by antagonists in this area informes how to utilise antagonists for optimal fire blight control [46].

Chapter 5 Final discussion

5.1 Colonisation of apple blossoms by *E. amylovora*

The research documented in this thesis has used three different analyses to demonstrate the colonisation process of *E. amylovora* on apple blossoms. The research has confirmed colonisation of the papillae cells by *E. amylovora* is a crucial step in the development of fire blight disease. The papillae cells hosted the highest population, as observed under the microscope and detected in the detached flower assay. The *E. amylovora* number on apple blossoms is correlated with risk of fire blight disease therefore minimising populations of *E. amylovora* on papillae cells is a promising target for disease prevention [25,184]. Iron limitation is suggested to have a role in *E. amylovora* pathogenicity. Papillae cells with both limited iron availability as well as secreted amino acids and sugars provide a surface that allows high population density and promotes pathogenicity [1,35–38,61,62,65]. Identification of effective antagonists should be focussed on strains that rapidly colonise the papillae cells as these are expected to prevent *E. amylovora* from accessing secreted nutrients, thereby preventing growth to large population sizes.

5.2 Addressing my hypotheses

The first hypothesis tested in this research is:

1) The four strains selected for this study will show an effect in inhibiting the growth of *E. amylovora*.

Hypothesis 1 was addressed in Chapters 2, 3, and 4 where data shows *P. vagans*, *P. agglomerans*, *P. fluorescens* and *S. melonis* all showed an effect in inhibiting growth of *E.*

amylovora in the double layer assay. The results of the detached flower assay indicated that only *S. melonis* was able to inhibit growth on apple blossom stigma and style when applied with *E. amylovora*. On the apple blossom stigma and style, *P. vagans* was able to prevent migration towards the hypanthium despite having no effect on the population size while no inhibition by *P. agglomerans* and *S. melonis* was observed. Based on these results, both *P. vagans* and *S. melonis* showed an effect in preventing growth of *E. amylovora* on the apple blossom through preventing growth to new areas and decreasing the CFU respectively.

The second hypothesis for this hypothesis is:

2) On any given surface, both artificial and plant, preemptive colonization will determine antagonistic success.

Hypothesis 2 was addressed in Chapters 2, 3, and 4 in which the double layer assay showed that all antagonists tested were able to prevent the growth of *E. amylovora*, but only *P. vagans* and *P. agglomerans* were also able to affect existing growth. The detached flower assay demonstrated that both *P. vagans* and *P. agglomerans* had no effect on isolated CFU of *E. amylovora* on the stigma and style, although *P. vagans* was able to prevent migration towards the hypanthium. *S. melonis* was associated with a decrease in CFU of isolated *E. amylovora* from the stigma and style. Populations of *E. amylovora* on the hypanthium were found to be increased when blossoms were also inoculated with *P. vagans* and *P. agglomerans*, compared to populations inoculated with *S. melonis* where no change was observed. This data shows that preemptive colonisation is not required for growth of *E. amylovora* to be affected by an antagonist. Despite co- inoculation with *E. amylovora*, both *P. vagans* and *S. melonis* were able to interfere with population size, or migration of *E. amylovora*.

The third hypothesis for this hypothesis is:

3) *E. amylovora* will impair the growth of antagonistic and potentially antagonistic strains when applied to surfaces, both artificial and plant.

Hypothesis 3 was addressed in Chapters 2, 3, and 4, where *E. amylovora* was found to prevent growth of *P. vagans*, *P. agglomerans*, *P. fluorescens*, and *S. melonis* in the double layer assay. Data in Chapters 2, 3 and 4 found that *E. amylovora* had no effect on *P. vagans* and *P. agglomerans* population sizes on the stigma and style, although the pathogen and antagonists segregated on the stigmas. The population of *S. melonis* was significantly decreased after 72 hours of incubation when co inoculated with *E. amylovora*. Populations of *P. vagans*, *P. agglomerans* and *S. melonis* isolated from the hypanthium were increased when blossoms were inoculated with *E. amylovora*. My research suggests that *E. amylovora* was able to impair growth of *S. melonis* but had no effect on *P. vagans* or *P. agglomerans* populations on apple blossom stigma and style. However inoculation of antagonists with *E. amylovora* was associated with an increase in hypanthium populations of the antagonists, which does not support my hypothesis. I conclude that antagonist and *E. amylovora* interactions are affected by the plant or media surface on which they are present, contributing to differing observations regarding antagonist and pathogen activity through Chapters 2, 3 and 4.

The fourth hypothesis for this hypothesis is:

4) Strains with an antagonistic effect against *E. amylovora* will colonise apple blossoms in the same way as the pathogen.

Hypothesis 4 was addressed in Chapter 4. *P. vagans* as a known antagonist did not colonise the apple blossom stigma the same way as *E. amylovora* but demonstrated an effect in preventing style migration by *E. amylovora*. The *P. agglomerans* colonisation process of the stigma is the same as *E. amylovora*, but *P. agglomerans* did not show any effect in inhibiting

E. amylovora growth. *S. melonis* despite effects in decreasing the stigma and style population of *E. amylovora* as detected in Chapter 3, showed a different colonisation process to the pathogen. In contrast with previous findings which found similarity of colonisation by antagonists is associated with greater antagonism, my research has not supported this hypothesis [37,96].

5.3 Future research to optimise antagonism for efficient biocontrol of fire blight

As a biocontrol strain for fire blight *S. melonis* or strains like *S. melonis* merit further research. *S. melonis* on the apple blossom showed very different activity than *P. vagans,* a known antagonist of *E. amylovora*. The detached flower assay found *S. melonis* populations decreased after 72 hours when inoculated with *E. amylovora*, but the microscopy showed an increase in the area of the papillae cells colonised by *S. melonis*. These observations are attributed to the formation of biofilms by *S. melonis* which protected *S. melonis* being washed off the plant surface and detected by the detached flower assay [185]. While the biofilm formation may offer protection to *E. amylovora*, reducing the number of *E. amylovora* cells which can be removed from the stigma and style by rain and dew. This in turn would decrease the number of pathogen cells that reach the hypanthium thereby decreasing the occurrence of fire blight [25,47].

The research demonstrates how microscopy investigations generating high spatial resolution images contribute towards an in depth understanding of antagonist action against *E. amylovora* on apple blossoms. Using a single- cell approach has additional merit for biocontrol research as demonstrated in this research by advancing understanding why pre emptive colonisation of antagonists is such a limiting factor in biocontrol of fire blight.

Microcopy data furthered interpretation of the detached flower assay results and how strains similar to *S. melonis* may have potential as fire blight biocontrol methods.

Antagonist efficiency could be improved by developing strains with greater papillae cell colonisation efficiency. Population size of *E. amylovora* is correlated with the risk of fire blight disease and the papillae cells with secreted amino acids, sugars, and iron were found to host the most dense populations of *E. amylovora* [1,35–38]. Preemptive exclusion of *E. amylovora* from these sites would minimise the population size, and therefore the risk of fire blight infection. *P. vagans* was able to prevent migration of *E. amylovora* towards the hypanthium, but as rain and humidity facilitate movement of *E. amylovora* this effect is unlikely to be sufficient to prevent fire blight disease [1,25,45]. Biocontrol strains capable of rapidly colonising the papillae cells may exhibit effective biocontrol, even when applied at the same time as *E. amylovora*. If further work supported this hypothesis, pressure on apple producers to apply biocontrol strains prior to seasons with high risk of fire blight, and reliance on Streptomycin as a fire blight control method would be reduced [72,83,84,186].

In the development of highly efficient colonising strains consideration should be given to whether high densities of antagonists on papillae cells would affect pollination by bees. The effect of small populations is expected to be minimal as the stigma is commonly colonised by a variety of species. However dense populations that would offer thorough fire blight protection may promote papillae cell collapse, thereby preventing pollination and in turn decreasing the apple agricultural output [33].

My research has shown that fire blight is a complex plant disease and evaluating antagonists for biocontrol should focus on those that efficiently colonise the papillae cells, using microscopy as an important tool for gaining additional information regarding antagonist and *E. amylovora* activity on the apple blossom surface.

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Supplementary data

6.1 *E. amylovora* control data

All of the *E. amylovora* data was compared using graphs and statistical analysis to test the reproducibility of the detached flower assay. All replicates are displayed on the following graphs, with each replicate set of 5 blossoms shown separately.

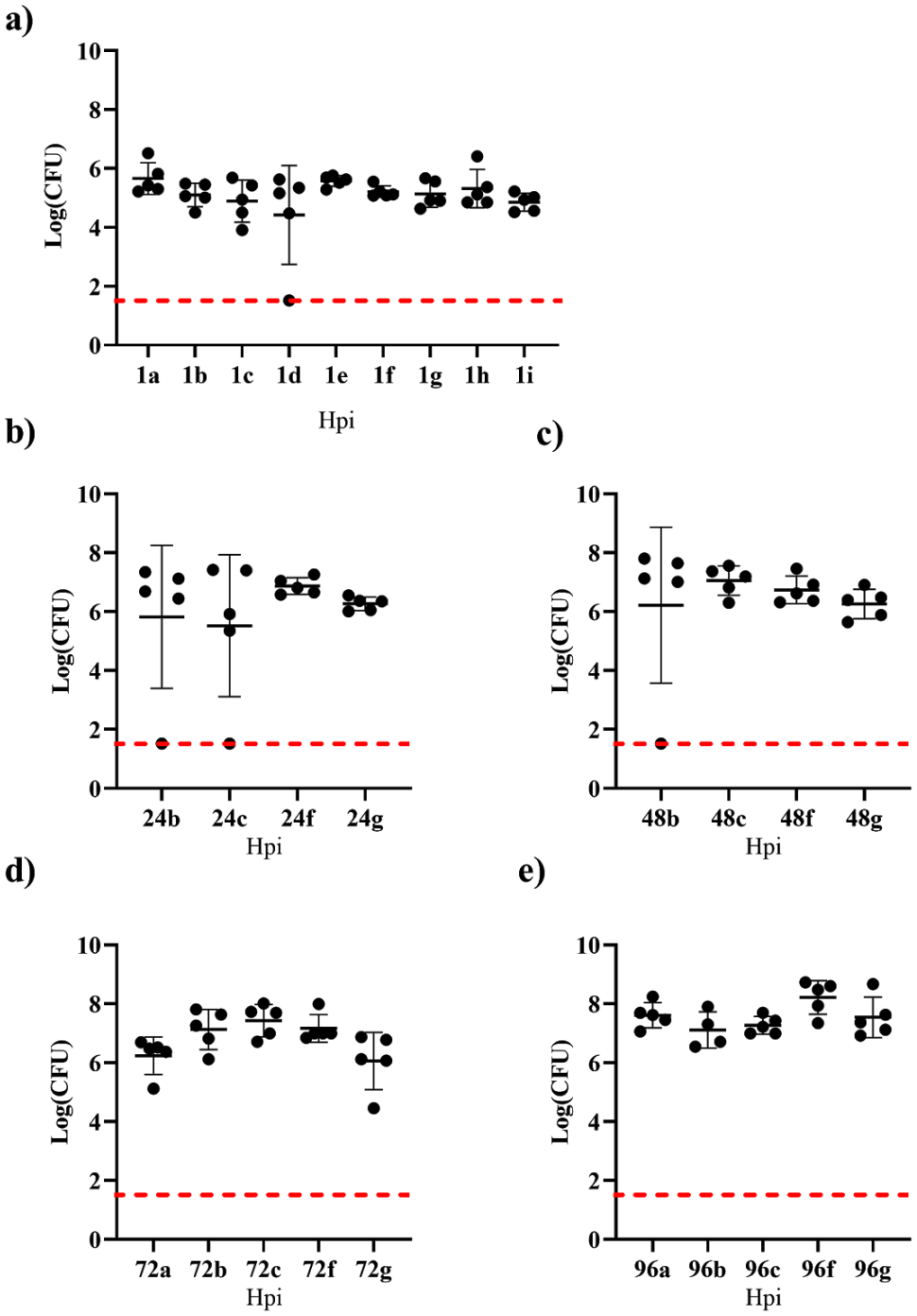

Figure 1. CFU of *E. amylovora* isolated from the apple blossom stigma and styles. Replicates of 5 are separated. The experiments are separated based on which antagonist assays they were generated from; a are from *P. vagans* assays, b, c, d, e are from *P. agglomerans* assays, f, g, h, i are from *S. melonis* assays.

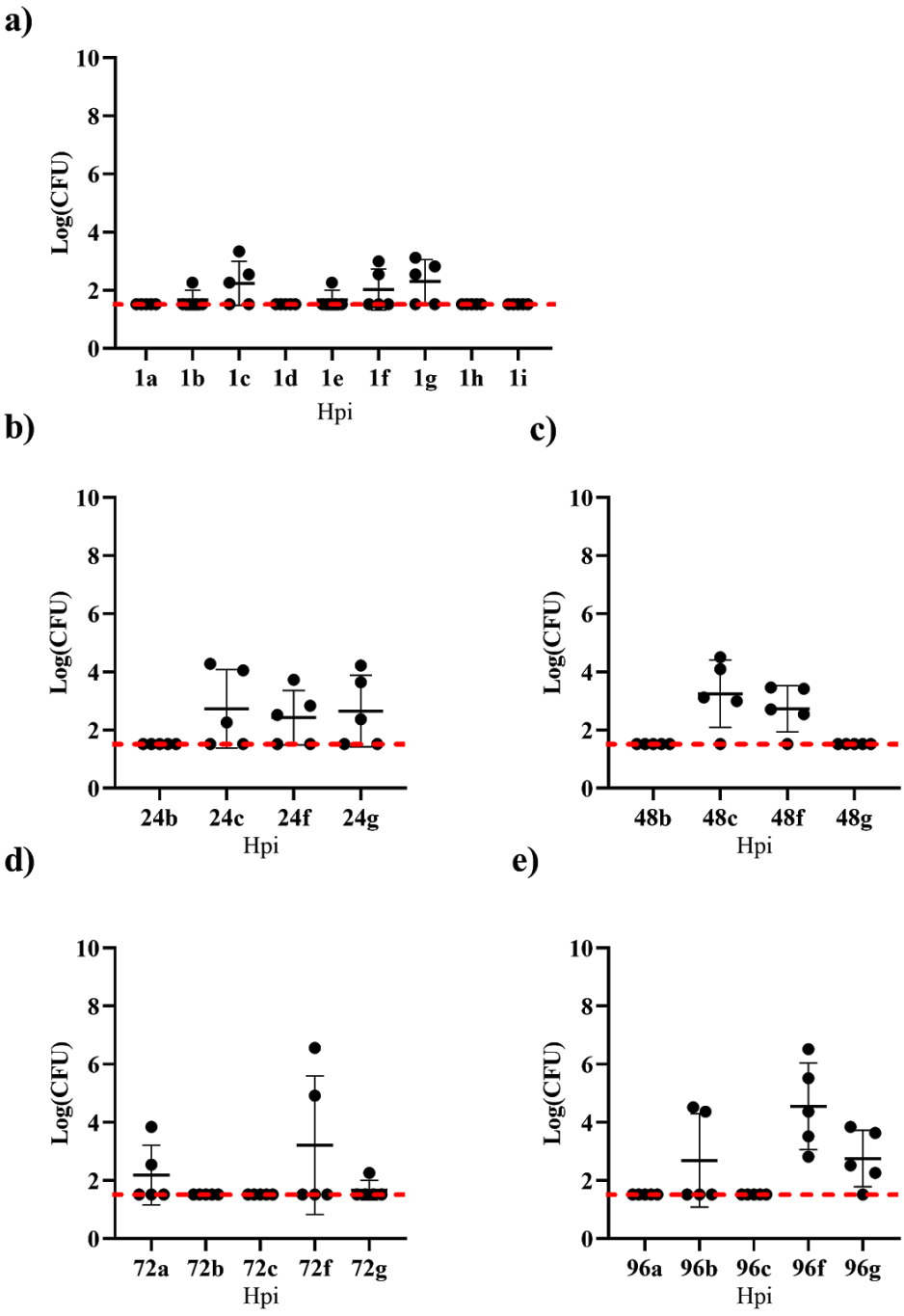

Figure 2. CFU of *E. amylovora* isolated from the apple blossom hypanthium. Replicates of 5 are separated. The experiments are separated based on which antagonist assays they were generated from; a are from *P. vagans* assays, b, c, d, e are from *P. agglomerans* assays, f, g, h, i are from *S. melonis* assays.

A one- way ANOVA test was used to compare the replicate control assays. This test was performed assuming a Gaussian distribution, but not assuming equal variances therefore the Welch's ANOVA test was selected. The one- way ANOVA tests the hypothesis: there is no significant difference in the population means between replicate sets of 5 blossoms.

Table 1. The results of the Welch's one- way ANOVA test used to compare replicates *E. amylovora* control assays. As the CFU isolated from the stigma frequently has all data points from a replicate set at the limit of detection therefore there was insufficient variation within the set to use the one- way ANOVA.

	1 hr	24 hrs	48 hrs	72 hrs	96 hrs
Stigma	0.0382	0.0441	0.2116	0.0589	0.1072
Hypanthium	-	-	-	-	-

Populations isolated from the stigma and style were at the majority of incubations times consistent throughout all replications and showed low variation within each set of five blossoms shown by Figure 1 and table 1. In comparison CFU isolated from the hypanthium showed much greater variation but could not be statistically compared because some data sets have all data points at the limit of detection (Figure 2). The analyses show the detached flower assay is a robust and consistent method for measuring bacterial strains on apple blossoms. Strains isolates from the hypanthium are expected to show greater variation, so any small changes to populations when changing inoculation time or bacterial strains inoculated are less likely to be detected.

6.2. *P. vagans* and *E. amylovora*

6.2.1 Normality plots

Normality analysis is presented for each of the representative blossom sets used to calculate the two- way ANOVA test, the results of this test are described in Chapter 3, section 3.3.2. The normality test was performed using the D' Agostino- Pearson omnibus normality test with $\alpha = 0.05$. This test investigated how normally distributed each set of 5 replicate blossoms in a single data set is. The results for each inoculation treatment are displayed in a single graph, but analyses of different incubation times is performed separately. A set of five is not sufficient for a mathematical calculation therefore QQ plots have been used to show this data.

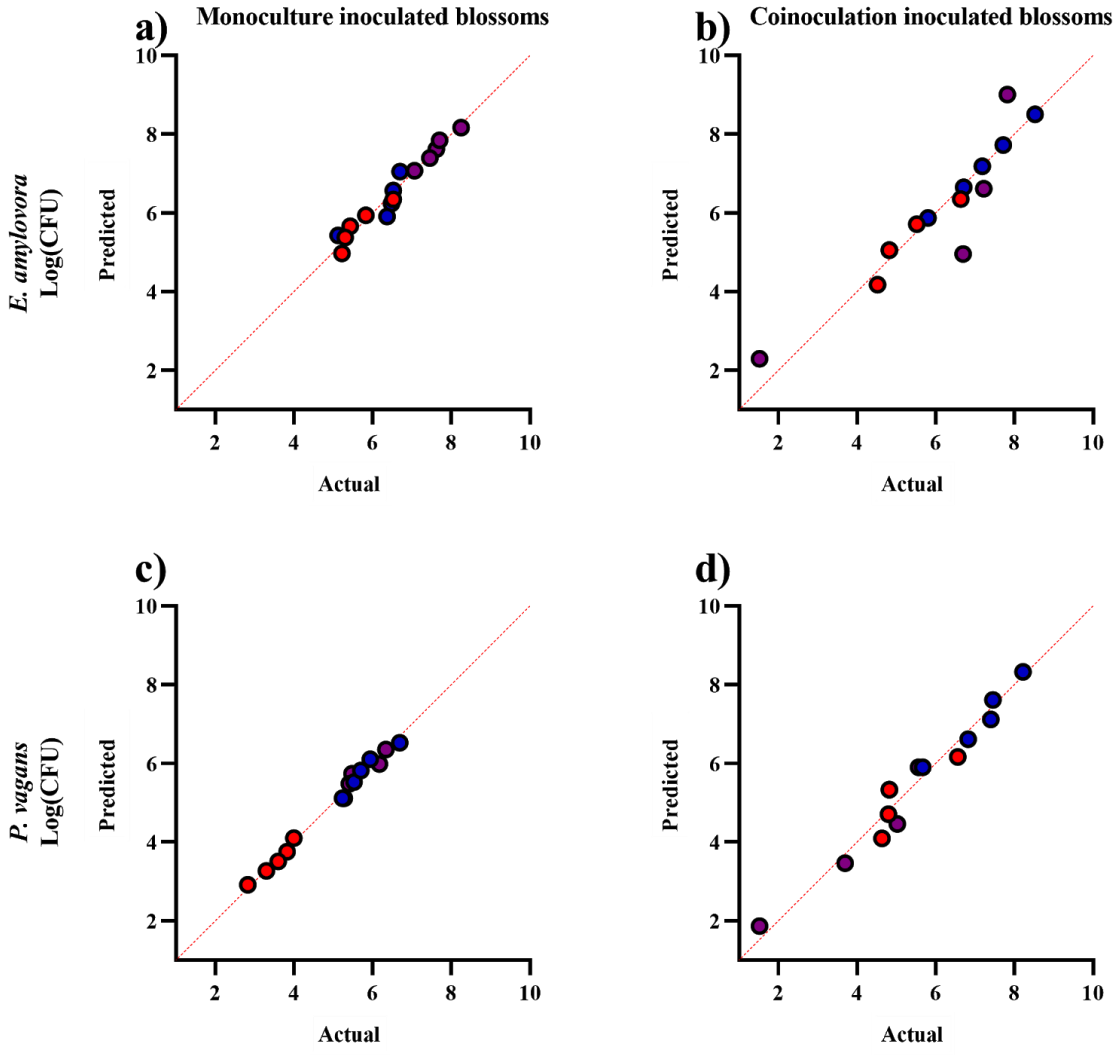

Figure 3. Isolated CFU from apple blossom stigma and style compared to the predicted values. Red data points indicate CFU isolation after 1 hr of incubation, blue data points indicate CFU isolation after 72 hrs of incubation, purple data points indicate CFU isolation after 96 hrs of incubation.

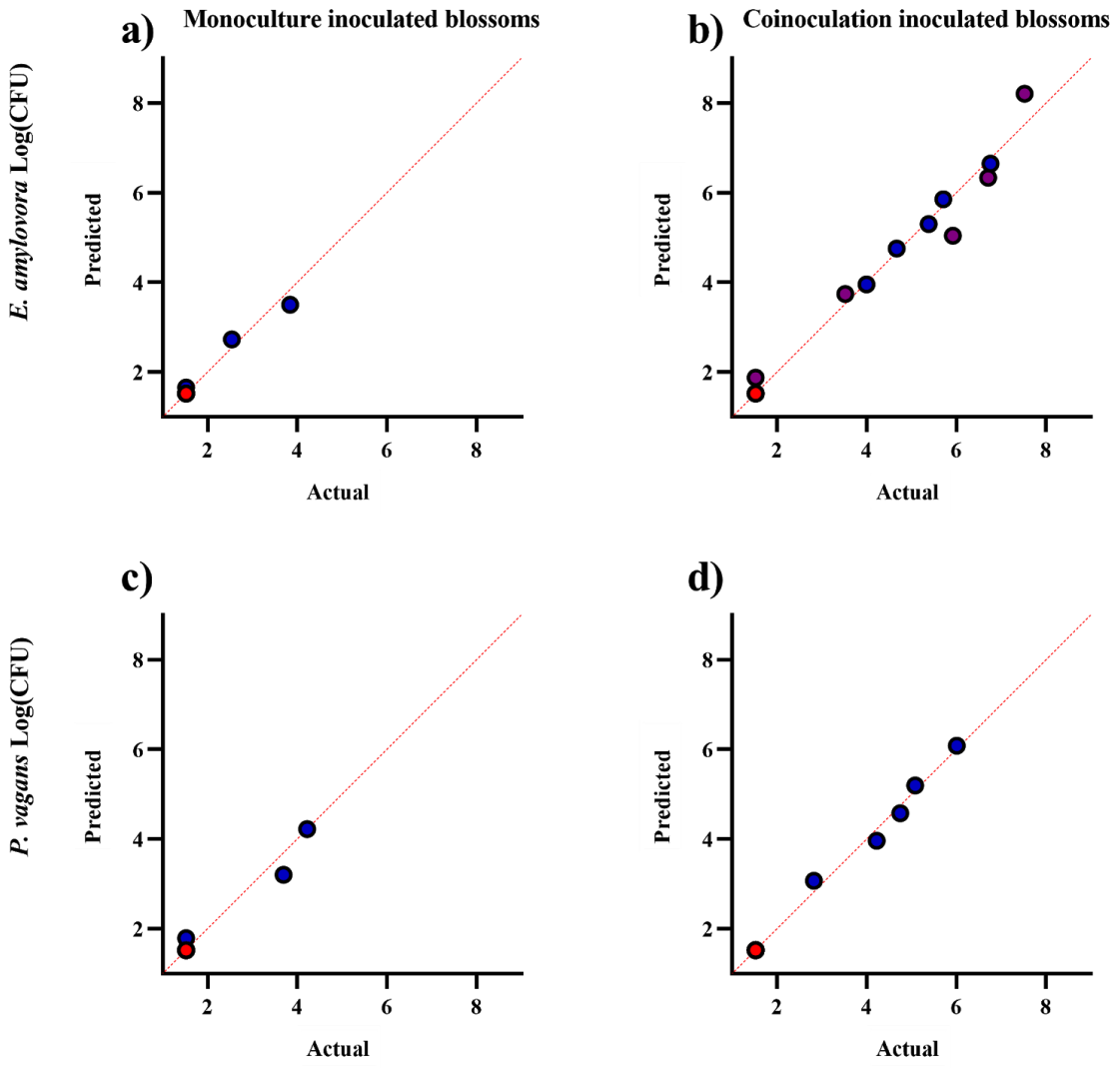

Figure 4. Isolated CFU from apple blossom hypanthium compared to the predicted values. Red data points indicate CFU isolation after 1 hr of incubation, blue data points indicate CFU isolation after 72 hrs of incubation, purple data points indicate CFU isolation after 96 hrs of incubation.

The representative blossom sets used for the two- way ANOVA analysis are shown in Figures 3 and 4 to be normally distributed therefore assuming normal distribution for statistical analyses is appropriate.

6.2.2 Results of the Welch's two- way ANOVA test

The two- way ANOVA analyses tests three hypotheses;

1) **the means of populations incubated for different lengths of time is the same**
2) **the means of populations with different inoculation treatments is the same**
3) **there is no interaction between the incubation time and inoculation treatment.**

A p- value less than 0.05 is evidence to support the alternate hypothesis that populations means are different when populations are subject to a different incubation time or incubation treatment. The two- way ANOVA analysis was used to compare populations of *E. amylovora* isolated after inoculation as a monoculture with populations isolated after inoculation with *P. vagans*. The two- way ANOVA analysis was also used to compare populations of *P. vagans* isolated after inoculation as a monoculture with populations isolated after inoculation with *E. amylovora*. The hypothesis of interest for my research is whether the population mean is different when blossoms are inoculated with a secondary strain compared to inoculation as a monoculture.

Table 2. The two- way ANOVA data output from analysis of *E. amylovora* and *P. vagans* populations. Columns labelled Incubation time show the p- value resulting from testing hypothesis 1. Columns labelled Inoculation treatment show the p- value resulting from testing hypothesis 2. Columns labelled Interaction between factors show the p- value resulting from testing hypothesis 3. The row labelled *E. amylovora* tested these

hypotheses on populations of *E. amylovora*, the row labelled *P. vagans* tested these hypotheses on populations of *P. vagans*.

	CFU isolated from the stigma/ style			CFU isolated from the hypanthium		
	Incubation Time	Inoculation treatment	Interaction between factors	Incubation Time	Inoculation treatment	Interaction between factors
E. amylovora	0.1397	0.3124	0.0173	0.0040	0.0012	0.0062
P. vagans	0.0004	0.6371	0.0002	0.0010	0.0316	0.0075

The two- way ANOVA analysis showed incubation time is associated with significantly different populations of *P. vagans* on the apple blossom stigma and style. Incubation time and inoculation treatment were associated with difference in populations of both *E. amylovora* and *P. vagans* on the apple blossom hypanthium. Interaction between incubation time and inoculation treatment was detected for both strains on the stigma/ style and the hypanthium.

6.3 *P. agglomerans* and *E. amylovora*

6.3.1 Normality plots

Normality analysis is presented for each of the representative blossom sets used to calculate the two- way ANOVA test, the results of this test are described in Chapter 3, section 3.3.3. The normality test was performed using the D' Agostino- Pearson omnibus normality test with α= 0.05. This test investigated how normally distributed each set of 5 replicate blossoms in a single data set is. The results for each inoculation treatment are displayed in a single graph, but the differing incubation times are not analysed together. A set of five is not sufficient for a mathematical calculation therefore QQ plots have been used to show this data.

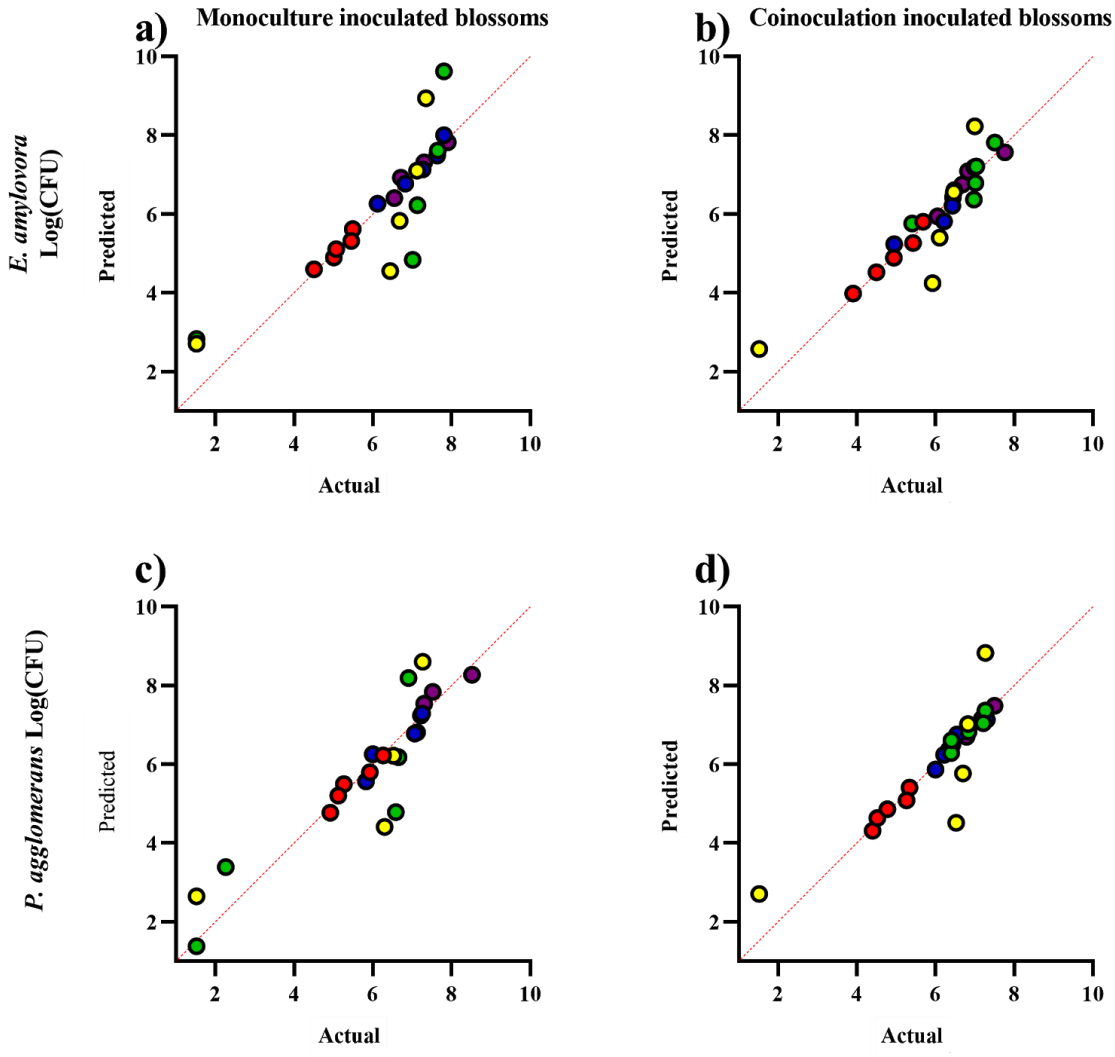

Figure 5. Isolated CFU from apple blossom stigma and style compared to the predicted values. Red data points indicate CFU isolation after 1 hr of incubation, yellow data points indicate CFU isolation after 24 hrs of incubation, green data points indicate CFU isolation after 48 hrs of incubation, blue data points indicate CFU isolation after 72 hrs of incubation, purple data points indicate CFU isolation after 96 hrs of incubation.

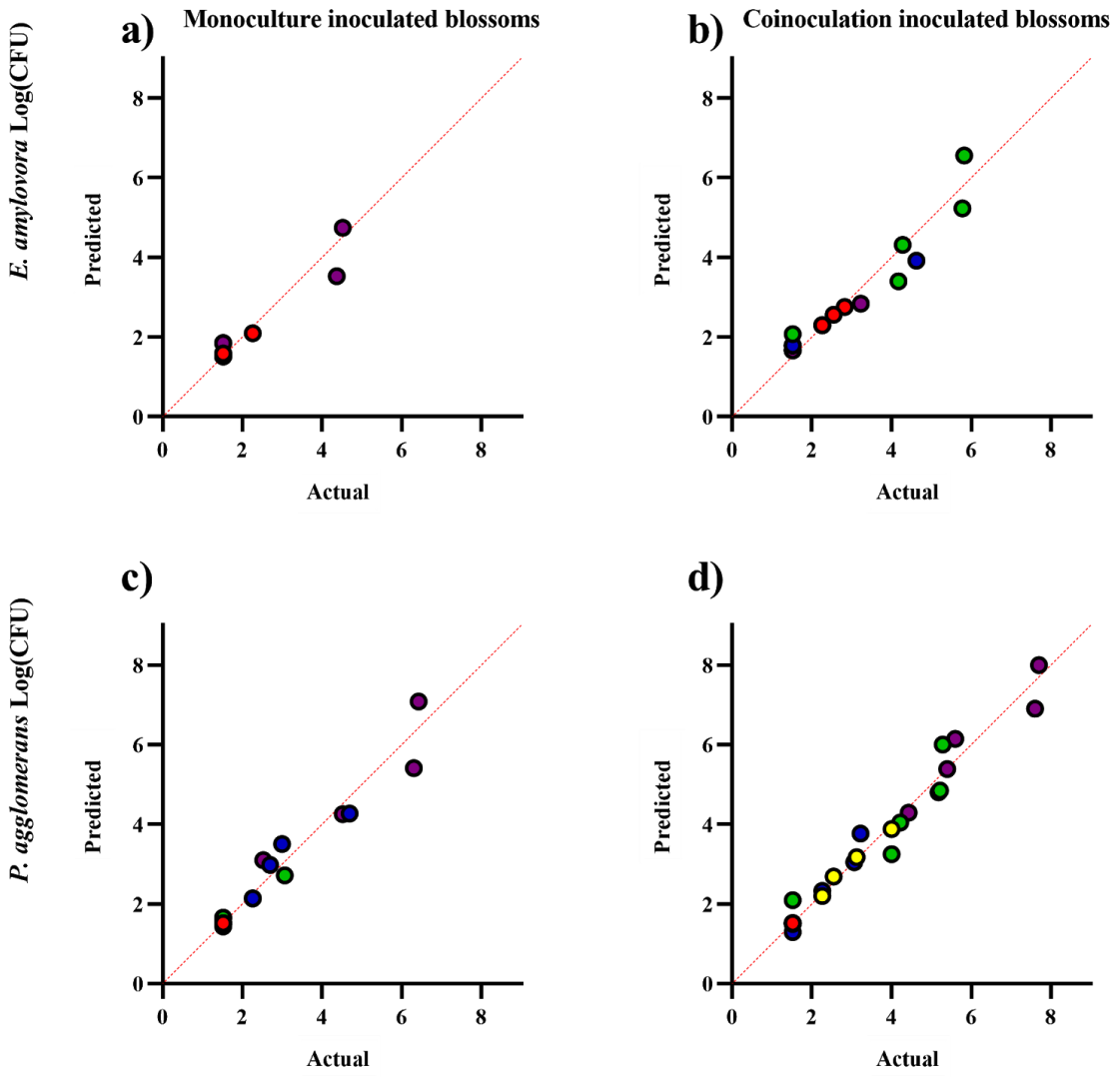

Figure 6. Isolated CFU from apple blossom hypanthium compared to the predicted values. Red data points indicate CFU isolation after 1 hr of incubation, yellow data points indicate CFU isolation after 24 hrs of incubation, green data points indicate CFU isolation after 48 hrs of incubation, blue data points indicate CFU isolation after 72 hrs of incubation, purple data points indicate CFU isolation after 96 hrs of incubation.

The representative blossom sets used for the two- way ANOVA analysis are shown in Figures 5 and 6 to be normally distributed therefore assuming normal distribution for statistical analyses is appropriate.

6.3.2 Results of the two- way ANOVA test

The two- way ANOVA analyses tests three hypotheses;

4) **the means of populations incubated for different lengths of time is the same**
5) **the means of populations with different inoculation treatments is the same**
6) **there is no interaction between the incubation time and inoculation treatment.**

A p- value less than 0.05 is evidence to support the alternate hypothesis that populations means are different when populations are subject to a different incubation time or incubation treatment. The two- way ANOVA analysis was used to compare populations of *E. amylovora* isolated after inoculation as a monoculture with populations isolated after inoculation with *P. agglomerans*. The two- way ANOVA analysis was also used to compare populations of *P. agglomerans* isolated after inoculation as a monoculture with populations isolated after inoculation with *E. amylovora*. The hypothesis of interest for my research is whether the population mean is different when blossoms are inoculated with a secondary strain compared to inoculation as a monoculture.

Table 3. The two- way ANOVA data output from analysis of *E. amylovora* and *P. agglomerans* populations. Columns labelled Incubation time show the p- value resulting from testing hypothesis 1. Columns labelled Inoculation treatment show the p- value resulting from testing hypothesis 2. Columns labelled Interaction between factors show the p- value resulting from testing hypothesis 3. The row labelled *E. amylovora* tested these hypotheses on populations of *E. amylovora*, the row labelled *P. agglomerans* tested these hypotheses on populations of *P. agglomerans*.

	CFU isolated from the stigma/ style			CFU isolated from the hypanthium		
	Incubation Time	Inoculation treatment	Interaction between factors	Incubation Time	Inoculation treatment	Interaction between factors
E. amylovora	0.0244	0.6451	0.8743	0.0367	0.0325	0.0006
P. agglomerans	0.0112	0.7827	0.2008	<0.0001	0.0268	0.0475

The two way ANOVA analysis showed that incubation time is associated with different population means of both *E. amylovora* and *P. agglomerans* isolated from the stigma and style. Populations of *E. amylovora* and *P. agglomerans* isolated from the hypanthium were different when subject to different inoculation treatments and incubation times. Populations isolated from the hypanthium were found to have interaction of incubation time and inoculation treatment.

3.4 *S. melonis* and *E. amylovora*

6.4.1 Normality plots

Normality analysis is presented for each of the representative blossom sets used to calculate the two- way ANOVA test, the results of this test are described in Chapter 3, section 3.3.4. The normality test was performed using the D' Agostino- Pearson omnibus normality test with α= 0.05. This test investigated how normally distributed each set of 5 replicate blossoms in a single data set is. The results for each inoculation treatment are displayed in a single graph, but the differing incubation times are not analysed together. A set of five is not sufficient for a mathematical calculation therefore QQ plots have been used to show this data.

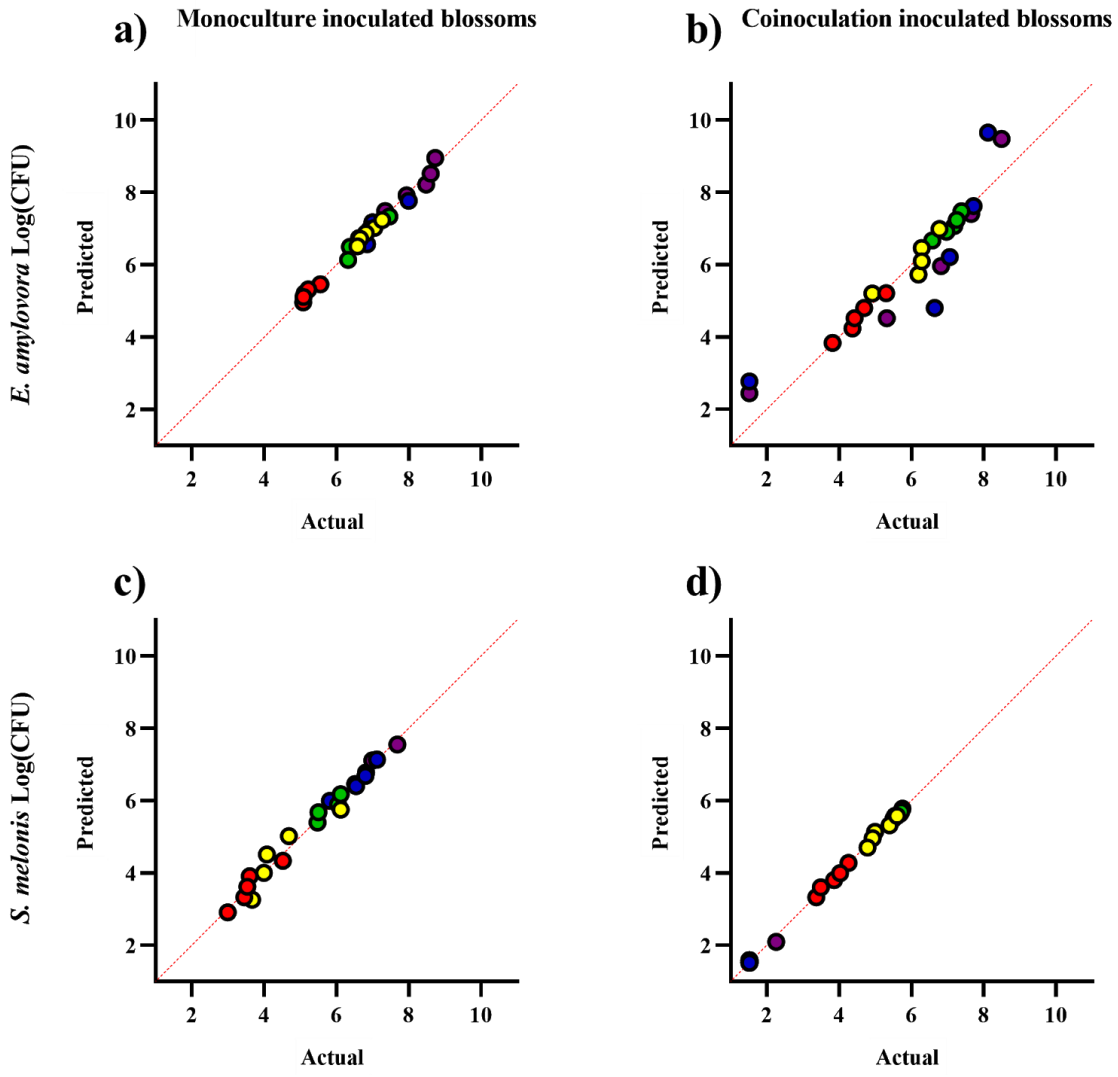

Figure 7. Isolated CFU from apple blossom stigma and style compared to the predicted values. Red data points indicate CFU isolation after 1 hr of incubation, yellow data points indicate CFU isolation after 24 hrs of incubation, green data points indicate CFU isolation after 48 hrs of incubation, blue data points indicate CFU isolation after 72 hrs of incubation, purple data points indicate CFU isolation after 96 hrs of incubation.

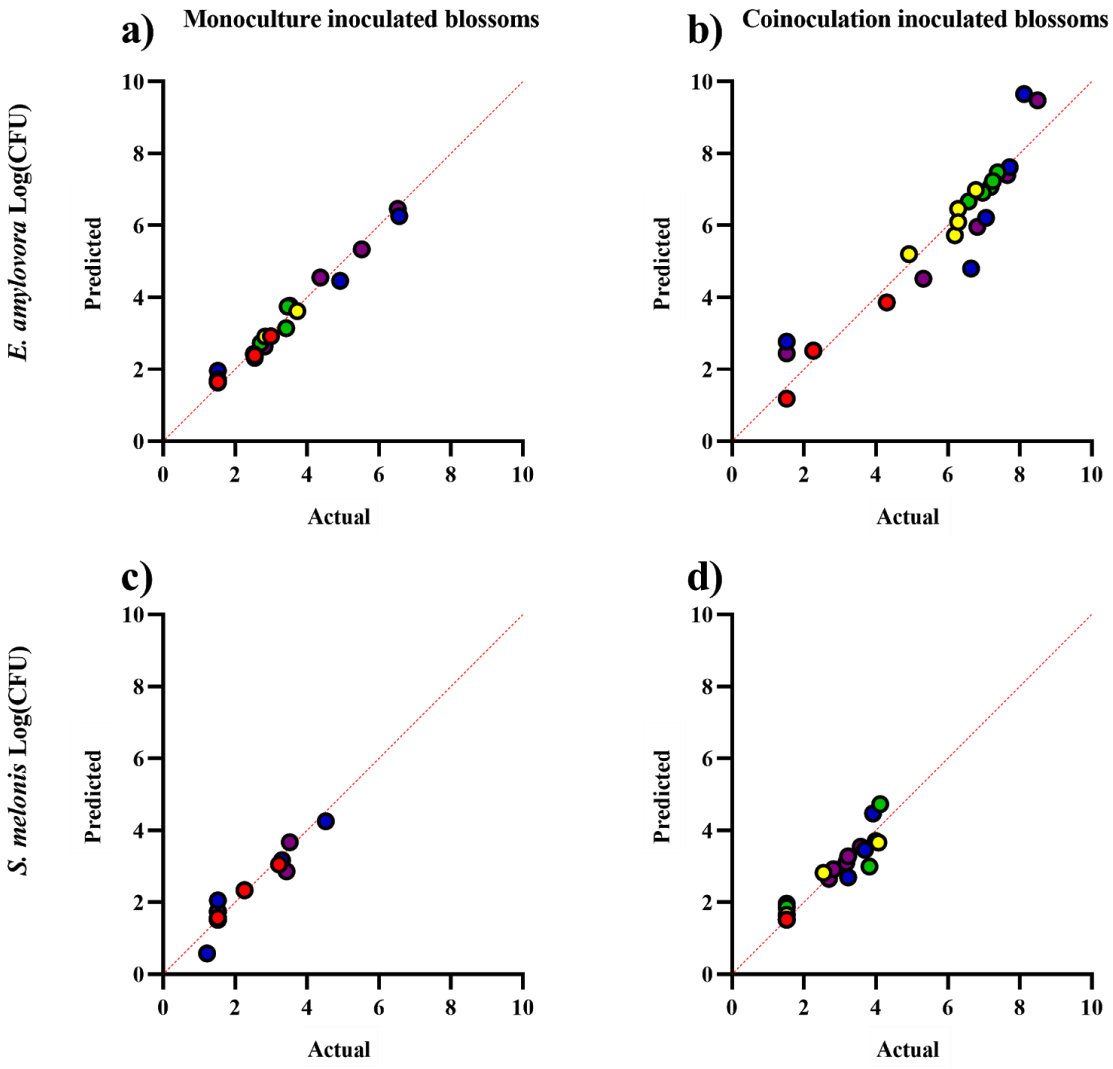

Figure 8. Isolated CFU from apple blossom hypanthium compared to the predicted values. Red data points indicate CFU isolation after 1 hr of incubation, yellow data points indicate CFU isolation after 24 hrs of incubation, green data points indicate CFU isolation after 48 hrs of incubation, blue data points indicate CFU isolation after 72 hrs of incubation, purple data points indicate CFU isolation after 96 hrs of incubation.

The representative blossom sets used for the two- way ANOVA analysis are shown in Figures 7 and 8 to be normally distributed therefore assuming normal distribution for statistical analyses is appropriate.

6.4.2 Results of the two- way ANOVA test

The two- way ANOVA analyses tests three hypotheses;

7) **the means of populations incubated for different lengths of time is the same**
8) **the means of populations with different inoculation treatments is the same**
9) **there is no interaction between the incubation time and inoculation treatment.**

A p- value less than 0.05 is evidence to support the alternate hypothesis that populations means are different when populations are subject to a different incubation time or incubation treatment. The two- way ANOVA analysis was used to compare populations of *E. amylovora* isolated after inoculation as a monoculture with populations isolated after inoculation with *S. melonis*. The two- way ANOVA analysis was also used to compare populations of *S. melonis* isolated after inoculation as a monoculture with populations isolated after inoculation with *E. amylovora*. The hypothesis of interest for my research is whether the population mean is different when blossoms are inoculated with a secondary strain compared to inoculation as a monoculture.

Table 4. The two- way ANOVA data output from analysis of *E. amylovora* and *S. melonis* populations. Columns labelled Incubation time show the p- value resulting from testing hypothesis 1. Columns labelled Inoculation treatment show the p- value resulting from testing hypothesis 2. Columns labelled Interaction between factors show the p- value

resulting from testing hypothesis 3. The row labelled *E. amylovora* tested these hypotheses on populations of *E. amylovora*, the row labelled *S. melonis* tested these hypotheses on populations of *S. melonis*.

	CFU isolated from the stigma/ style			CFU isolated from the hypanthium		
	Incubation Time	Inoculation treatment	Interaction between factors	Incubation Time	Inoculation treatment	Interaction between factors
E. amylovora	0.0095	0.0129	0.3021	0.0161	0.9458	0.2960
S. melonis	<0.0001	<0.0001	<0.0001	0.0049	0.0676	0.2470

The two- way ANOVA analysis showed that on the stigma and style, both *E. amylovora* and *S. melonis* were affected by incubation time and inoculation treatment. On the hypanthium, only incubation time was associated with a difference in population mean for both *E. amylovora* and *S. melonis*.